

**Investigating the role of IL-4/IL-13 signalling
through the IL-4 receptor alpha (IL-4R α) on
keratinocytes
in murine models of
Leishmania major and *Schistosoma mansoni***

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Melissa Govender

GVNMEL010

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Supervisor: Prof. F. Brombacher

Co-supervisors: Dr. R. Hurdal and Dr. R. Guler

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“....with God everything is possible...” Matthew 19:26

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Abbreviations and symbols

ANOVA	Analysis of variance	iNOS	Inducible nitric oxide synthase
APCs	Antigen presenting cells	JAK	Janus tyrosine kinases
AP	Alkaline phosphatase	<i>L. major</i>	<i>Leishmania major</i>
APC	Allophycocyanin	LN	Lymph Node
BMDCs	Bone marrow-derived dendritic cells	MHC	Major histocompatibility complex
CAB	Chromotrope 2R & analine blue	MZ	Marginal zone B cell
CD	Cluster of differentiation	NETs	Neutrophil extracellular traps
CL	Cutaneous leishmaniasis	NK	Natural killer
Cre	Cyclization recombinase	OD	Optical density
DCs	Dendritic cells	PAMPS	Pathogen-associated molecular patterns
DMEM	Delbucco's Minimal Eagle's Medium	PAS	Periodic Acid Schiffs
ELISA	Enzyme-linked Immunosorbent Assay	PBS	Phosphate buffered saline
FACS	Fluorescence-activated cell sorter	PCR	Polymerase chain reaction
FCS	Fetal calf serum	PE	Phycoerythrin
FITC	Fluorescein isothiocyanate	PNP	4-Nitrophenylphosphate
FO	Follicular B cell	SEM	Standard error of the mean
$\gamma\delta$	Gamma delta T cells	SEA	Soluble egg antigen
GFP	Green fluorescent protein	SLA	Soluble <i>Leishmania</i> antigen
GM-CSF	Granulocyte macrophage-colony stimulating factor	<i>S. Mansoni</i>	<i>Schistosoma mansoni</i>
H&E	Haematoxylin and eosin	STAT	Signal transducing activators of transcription
HRP	Horseradish peroxidase	TCR	T cell receptor
IFN- γ	Interferon gamma	Th	T helper
Ig	Immunoglobulin	TGF	Transforming growth factor
IL	Interleukin	TNF	Tumor necrosis factor
ILC	inate lymphoid cell	Treg	T regulatory
IL-4R α	Interleukin-4 receptor-alpha		
IMDM	Iscove's Modified Dulbecco's Medium		

Abstract

Keratinocytes represent the major cell type in the skin. During cutaneous leishmaniasis (CL) and schistosomiasis, the skin is important during the parasite life cycle. While Th1 immunity is required to control CL, protection during schistosomiasis requires Th2 immunity. Paradoxically, Th2 characteristic IL-4 secreted early during *L. major* infection in mice, can drive a Th1 response by instructing dendritic cells to produce IL-12. Additionally, keratinocytes at the site of *L. major* infection in C57BL/6 mice, were postulated to be the source of the IL-4. We investigated if IL-4/IL-13 signalling via the IL-4R α on keratinocytes contributed to early immunity during CL and schistosomiasis. Keratinocyte-specific IL-4R α deficient (KRT14^{cre}IL-4R α ^{-lox}) BALB/c and C57BL/6 mice were generated by gene targeting and site-specific recombination (cre/loxP) under control of the *KRT14* locus. In the *L. major* footpad model, KRT14^{cre}IL-4R α ^{-lox} BALB/c mice developed increased swelling, high parasite burdens, and cytokine and antibody secretion similar to littermate controls. *L. major*-infected KRT14^{cre}IL-4R α ^{-lox} C57BL/6 mice had decreased footpad swelling, low parasite burdens, a dominant Th1 cytokine response, and low type 1 and 2 antibody titres, similar to littermate control and resistant C57BL/6. In the *L. major* ear model, KRT14^{cre}IL-4R α ^{-lox} BALB/c mice developed increased swelling, high parasite burdens, Th1 and Th2 cytokines, and high antibody titres, similar to littermate controls. *L. major* LV39-infected KRT14^{cre}IL-4R α ^{-lox} BALB/c mice showed significantly decreased parasite burdens in the ear, compared to littermate controls. *L. major*-infected KRT14^{cre}IL-4R α ^{-lox} C57BL/6 mice in the ear model, had decreased swelling, low parasite burdens, a dominant Th1 immune response, and low type 1 and 2 antibody titres, similar to littermate control and C57BL/6 mice. In the *Schistosoma* model, survival of *S. mansoni*-infected KRT14^{cre}IL-4R α ^{-lox} BALB/c mice was similar to littermate controls during mortality studies. During acute infection, *S. mansoni*-infected KRT14^{cre}IL-4R α ^{-lox} BALB/c mice showed gut pathology, hepatosplenomegaly, cytokine production, low type 1 and high type 2 antibodies, similar to littermate controls. In comparison to littermate controls, *S. mansoni*-infected KRT14^{cre}IL-4R α ^{-lox} BALB/c mice had smaller granulomas. Collectively, our results indicate that IL-4/IL-13 signalling through the IL-4R α on keratinocytes is not required for control during CL or acute schistosomiasis, but does contribute to efficient granuloma formation during acute schistosomiasis.

Chapter 1: Introduction and literature review

1.1 The immune system

The immune system represents the assortment of cells and proteins, collectively working together to protect the body and its contents from foreign, invading agents, including viruses and bacteria among others. Essentially, it is a mechanism of defence to successfully overcome pathogenic invasion. The immune system may be separated into two components: the innate and the adaptive immune response [1-3].

1.1.1 Innate immunity

The innate immune response is the first line of defence against invading pathogens. This response is non-specific and employed almost immediately (within minutes), or within the first few hours of having encountered the pathogen. There is no immunological memory, hence no recognition of the same pathogen during a re-exposure. The innate immune system functions to recruit specialised immune cells to the site of infection or inflammation [2-4].

The innate immune pathway includes anatomical and physiological barriers. Anatomical barriers comprise: the skin, which acts as mechanical barrier to protect against germs, the mucus membrane present in the respiratory and gastrointestinal tract to trap microorganisms, and the ciliary propulsion on epithelial cells in the lungs to eradicate microbes. Physiological barriers comprise: lysozyme secretion (including enzymes in lysozyme of tears), low pH, complement, antimicrobial peptides, specialised cells, and cytokines including interferons. During damage to tissue, an acute phase inflammatory response is elicited. The initial response causes the body temperature to be raised, followed by an increase in the production of various acute-phase proteins (C-reactive and mannan-binding proteins for e.g.), primarily by the liver. Some acute phase proteins bind molecules present on bacterial or fungal cell wall, through pattern recognition (C-reactive and serum amyloid proteins), while the mannan-binding lectin will bind to mannose sugar molecules not present on mammalian cells. While they are non-specific, acute phase proteins are responsible and important in directing phagocytic cells to the site of infection [1-4].

As mentioned above, the innate immune system comprises a range of specialised cells. Granulocyte cells contain densely staining granules within their cytoplasm, and includes the neutrophils, eosinophils, and basophils. The duration of their life span is a few days, however, they are produced in great numbers, in response to foreign agents. The neutrophils comprise

the largest group of cells, and are extremely important for the execution of the innate immune pathway. Neutrophils are phagocytic cells that eradicate the foreign agents by engulfing them and taking them into intracellular vesicles in their cytoplasm, which contain lysing enzymes and antimicrobial agents. Macrophages are also phagocytic cells, responsible for the killing of pathogens, but also being important in presenting foreign agents to the immune system [3, 4]. They recruit other phagocytic cells to the infection site by chemokine release. Classically activated macrophages or M1 macrophages, are induced by intracellular pathogens, which results in a nitric oxide-mediated killing inflammatory immune response. Conversely, alternatively activated macrophages or M2 macrophages, act in response to certain extracellular pathogens, and have important regulatory functions [5, 6]. Macrophages also play a role in allergic responses. Alveolar macrophages are found in the lung and are crucial in pulmonary immunity [7, 8].

Eosinophils and basophils are fewer in numbers when compared to neutrophils, and they release their enzymes and toxic proteins from their cytoplasmic granules, in response to invading agents, especially against parasitic agents. Basophils are non-phagocytic cells that release granules containing histamines, serotonin, and prostaglandins, and along with eosinophils and mast cells, are important in promoting allergic responses and of anti-parasitic immunity. Mast cells contain large granules in their cytoplasm, and upon activation, these are released, assisting the induction of inflammation. Eosinophils that release cytotoxic factors, are important for the eradication of pathogens that are too large to be phagocytosed [1-4].

Natural killer (NK) cells are large and also granulated, and are important in the identification and elimination of cells that have been infected with a virus, as well as tumor cells. They release cytotoxic granules, which contain cytotoxic granzymes and perforin (a pore forming protein) onto the target cells surface. This allows for granzymes to penetrate the cell membrane and trigger programmed cell death [1-4]

Dendritic cells (DCs) have long finger-like protrusions. There are different types of dendritic cells. The immature dendritic cells travel from the bone marrow to tissues, via the bloodstream. They are phagocytic cells, taking up and degrading pathogens. This causes the dendritic cells to become mature cells, which by presenting the resultant antigens from pathogen degradation, are capable of activating the adaptive immune T lymphocytes. It is for this reason, they are referred to as antigen presenting cells (APCs). Due to their function as messengers between the innate and adaptive immune pathways, DCs are extremely important [1-4].

1.1.2 Adaptive immunity

The adaptive immune pathway is triggered when the innate immune pathway cannot efficiently eradicate the foreign invading agent, which may cause an infection to occur. Essentially, the principal functions of this pathway is to: 1, recognise specific “non-self” antigens in the presence of “self” antigens; 2, generate pathogen-specific immunologic pathways that eradicate the specific foreign agent or pathogen-infected cells; and 3, to develop immunologic memory capable of efficiently and rapidly eradicating the specific pathogen in the event of re-infection [3, 9]. Compared to the innate immune pathway, adaptive immunity takes time to develop, and has greater specificity. This pathway employs two specialized groups of cells: T and B lymphocytes. As mentioned above, APCs may activate T and B cells by presenting a variety of antigens to randomly generated and unique antigen receptors on the surface of the cells. On the surface of APCs, are protein molecules called major histocompatibility complex (MHC). Two classes of MHC exists: class I, presenting intracellular peptides on all nucleated cells; and class II, presenting extracellular peptides on a group of immune cells, including DCs, B cells and macrophages. When cells become infected with a foreign agent, the MHC will present the pieces of the foreign antigen. During this time, costimulatory molecules that are expressed on APCs will engage receptors on T cells. Upon activation of the T cell receptor (TCR) on T cells by the APCs MHC-peptide complex, the T cell and DCs will secrete small proteins called cytokines (e.g. IL-4 and IFN- γ (T cell) or IL-12, and IL-18 (DCs), among others) to assist in the elicited immune response [1, 3, 4, 10]. As a result of antigen presentation, T cells may differentiate into a number of subtypes, discussed below in Section 1.1.4. Our study was, however, focussed mostly on two of these types: T helper (Th) cells (cell markers: CD3⁺CD4⁺), important in carrying out the immune response, by guiding a variety of cells required to elicit the response; and cytotoxic T (Tc) cells (cell markers CD3⁺CD8⁺), whose main purpose is to eradicate infected cells. While Th cells are activated by MHC class II-bound peptides, Tc cells are activated by MHC class I-bound peptides [3, 4].

1.1.3 Cytokines

Cytokines are a group of small soluble protein molecules (approximately 25 kDa) with pleiotropic functions, that are crucial in establishing an efficient immune response [1, 11, 12]. They are specialized proteins secreted by cells as a form of message or communication to other cells, so as to alter or affect them (paracrine), or in fact to alter or affect the secreting cell itself (autocrine). In order to send the intracellular signals, cytokines will bind to specific receptors on the surface of the cell. Cytokines do not have a very long half-life. They are produced by

almost every cell, and serve various purposes. Cytokines play a crucial role in numerous processes, including: cell activation and division; repair; apoptosis; cell differentiation and proliferation, among others. Different types of cytokines may be produced by different cells. Th1 cells secrete primarily interferon- γ (IFN- γ), IL-2, and tumor necrosis factor- β (TNF- β), while Th2 cells secrete primarily IL-4, IL-5, IL-6, IL-10 and IL-13 [1-4, 12, 13].

1.1.4 CD4⁺ T cell differentiation

T cell activation and differentiation is dependent on the environment, the type or mode of APC, co-stimulatory molecules, and cytokine production. All T cells express the CD3- antigen specific receptor, and depending on the pathogen encountered and the cytokine profile, once activated, they may differentiate into separate and distinct effector groups. [14-18]. The Th1 and Th2 immune pathways mentioned above are the two major classes of the T cell response, each class producing its unique sets of cytokines [3, 19, 20]. JAKs (janus tyrosine kinases) and STATs (signal-transducing activators of transcription) are families of proteins, whose intracellular signalling pathways may be activated by various cytokines. Differentiation to a Th1 pathway may be induced by the increased production of IL-12 via the STAT1/T-bet pathway. In a nutshell, Th1 characteristic cytokines including IFN- γ and IL-12, stimulate the JAK-STAT pathway, which results in the activation of certain genes. STAT1 and STAT4 are activated by innate immune cytokines during early infection, are important for the development of a Th1 response. STAT1 induces the expression of T-bet, a transcription factor, in activated CD4 T cells, switching on genes for the IFN- γ and the IL-12 receptor, and causing these cells to follow the Th1 lineage. Dendritic cells and macrophages secrete IL-12, activating STAT4, required for expansion and differentiation of the Th1 cells. The increased or primary production of the Th1 cytokines IFN- γ , IL-2 and IL-12 results from a positive feedback loop, and allows for the protection of the host against many intracellular pathogens. The IL-2 cytokine is important for cell proliferation, as well as the stimulation and cytotoxicity of CD8⁺ T cells. The Th1 response employs classically activated macrophages mentioned above, CD8⁺ T cells and natural killer cells for their cytotoxic functions, as well as protective IgG antibodies, and delayed-type hypersensitivity [1, 2, 21-27].

The Th2 pathway is brought about by IL-33 secretion of epithelial cells which activates a set of cells known as innate lymphoid cells (ILC), more specifically, ILC2, via the T1/ST2 receptor. The ILC2 cells respond by producing IL-4, which promotes the Th2 response via STAT6/GATA3 expression. Essentially, when T cell activation with antigen occurs, and it comes into contact with IL-4, STAT6 becomes activated by the receptor for IL-4 (IL-4R α).

STAT6 is important for the expression of GATA3, a transcription factor that activates genes for various Th2 produced cytokines, including IL-4 and IL-13, which are associated with helminth, asthma and allergic responses [1-4]. Th2 cytokines also include IL-5, IL-6, and IL-9. Th2 immunity promotes responses that are mediated by eosinophils, mast cells, and IgE antibodies. Essentially, a source of IL-4 may be produced by conventional T and B cells, basophils and eosinophils, among other cells, and this induces B cell class-switching to IgE. Th2 immunity also employs alternatively activated macrophages described above, mast cell proliferation, eosinophilic inflammation, goblet cell hyperplasia, and the production of excess mucous. During acute infections, there is a combined effort between both Th1 and Th2 responses, in contrast to chronic infection or allergic reactions, where either of the two responses may become dominant [3-9]. Due to inhibitory intracellular effects, Th1 and Th2 responses counter regulate each other. This is evident in the suppression of a Th2 polarised response by Th1-derived IL-12 and STAT1, and conversely, the prevention of Th1 cells from developing, by Th2-derived IL-10 and GATA-3 [19, 20].

Other T cell subsets exist, to carry out immune functions together with Th1 and Th2 responses [28]. Th17 cells are induced earlier on during the adaptive immune response, with their primary role being reported to defend against extracellular bacteria and fungi, occurring via the stimulation of neutrophils. A Th17 immune response progresses when T cells secrete IL-21, which acts on the same cells and activates STAT3 (transcription factor) which is important for the Th17 response. While the presence of IL-6 and transforming growth factor beta (TGF- β) are required for the production of Th17 cells, also required is the absence of IL-4 and IL-12 [1, 29-31]. TGF- β , which are Th3 lineage T cells, is important for the generation of CD4⁺CD25⁺FoxP3⁺ regulatory T cells (Treg), which are implicated in IL-10-linked immune-suppression to avert inflammatory responses that are dangerous [32, 33]. TGF- β is also responsible for re-programming Th2 T helper cells to remove their IL-4 cytokine profile, and replace it with IL-9, converting them to Th9 lineage [34-36]. A Th5 subset of T cells that produce IL-5 also exists, and is dependent on the signalling of IL-33 via the ST2 and Myd88 pathway [37, 38].

1.1.5 Keratinocytes

The skin is the first line barrier of protection in both humans and animals, and regarded as an immune organ. It contains various cells in the dermis and epidermis (stratified epithelium composed of 10-20 layers of keratinocytes), including dermal dendritic cells and epidermal Langerhans cell. The cell population, however, that comprises 90% of the cells found in the

epidermis, are the keratinocytes. Due to their abundance, a role for these cells in maintaining healthy and infection/wound-free skin should not be surprising [39].

There are 4 types of keratinocytes, based on both phenotypic and biochemical properties: basal; spinous; granular; and cornified keratinocytes. The classification of basal keratinocytes is based on their close contact with the basement membrane, their mitotic activity, and the expression of keratin (K) intermediate filaments, K5 and K14 [40]. While they do also begin the expression of the keratins K1 and K10, the earliest markers of terminal differentiation, these are only expressed fully, later in the upper spinous and granular layers [41, 42]. As the keratinocytes move through the epidermal layers, they switch from a state of proliferation (growing/producing in numbers) in the basal layer, to a state of differentiation. By the time they reach the cornified layer, they become flattened and dead (Figure 1.1) [43-45].

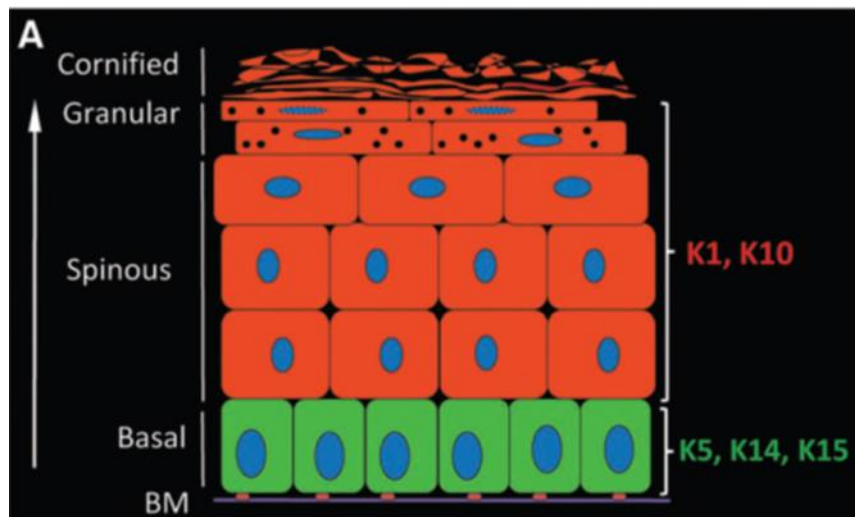


Figure 1.1 Schematic illustration of the structure of the epidermis. Major constituent keratinocytes are present at various levels, and characterised by keratin (K). Basal layer keratinocytes are in close proximity to the basement membrane (BM) and express K5, K14 and K15. As keratinocytes differentiate, they form the subsequent layers: spinous and granular; which express K1 and K10, and the cornified layer of dead cells. Image sourced from Pastar *et al.* [46].

In addition to barrier maintenance, keratinocytes are important for restoring the epidermis after injury during the process of epithelialization (covering shed/denuded epithelial surface). Wound healing is a normal biological process [47]. It involves various extracellular matrix components, soluble mediators, and resident cells, which include fibroblasts, nerve cells and keratinocytes, and subtypes of infiltrating leukocytes. All these components are involved in the process of wound healing, which, in early studies was characterised as a three phased process: inflammation; tissue formation; and tissue remodelling (Figure 1.2) [48, 49], but with further

research, is now characterised as a four phased process: haemostasis, inflammation, proliferation, and tissue remodelling/resolution (Figure 1.3) [50].

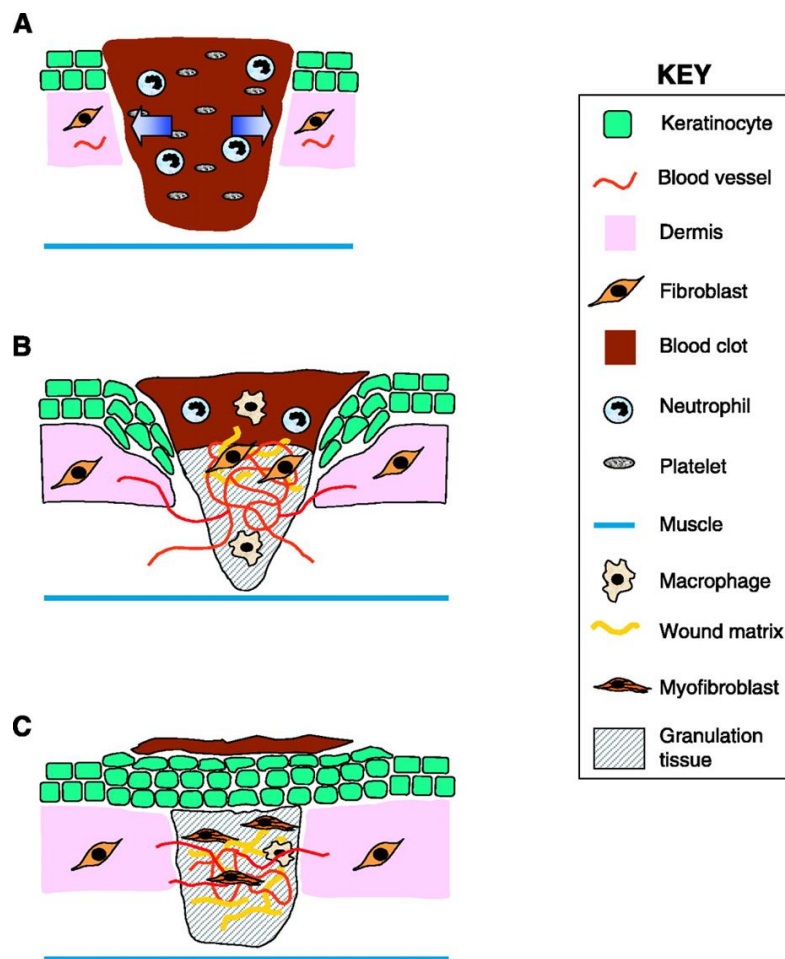


Figure 1.2 Schematic representation illustrating stages and cells involved during wound healing. (A) 12-24 hours after injury: wounded area is filled with a blood clot and neutrophils have invaded into the clot. (B) 3-7 days after injury: majority of neutrophils have undergone apoptosis, macrophages are now abundant in the wound tissue, endothelial cells migrate into the clot and proliferate to form new blood vessels, fibroblasts migrate into the wound tissue to proliferate and deposit extracellular matrix, keratinocytes proliferate at the wound edge and migrate down the injured dermis and above the provisional matrix. (C) 1-2 weeks after injury: the wound is completely filled with granulation tissue, fibroblasts have transformed into myofibroblasts, leading to wound contraction and collagen deposition. The wound is completely covered with a neoepidermis. Image sourced from Werner and Grose [51].

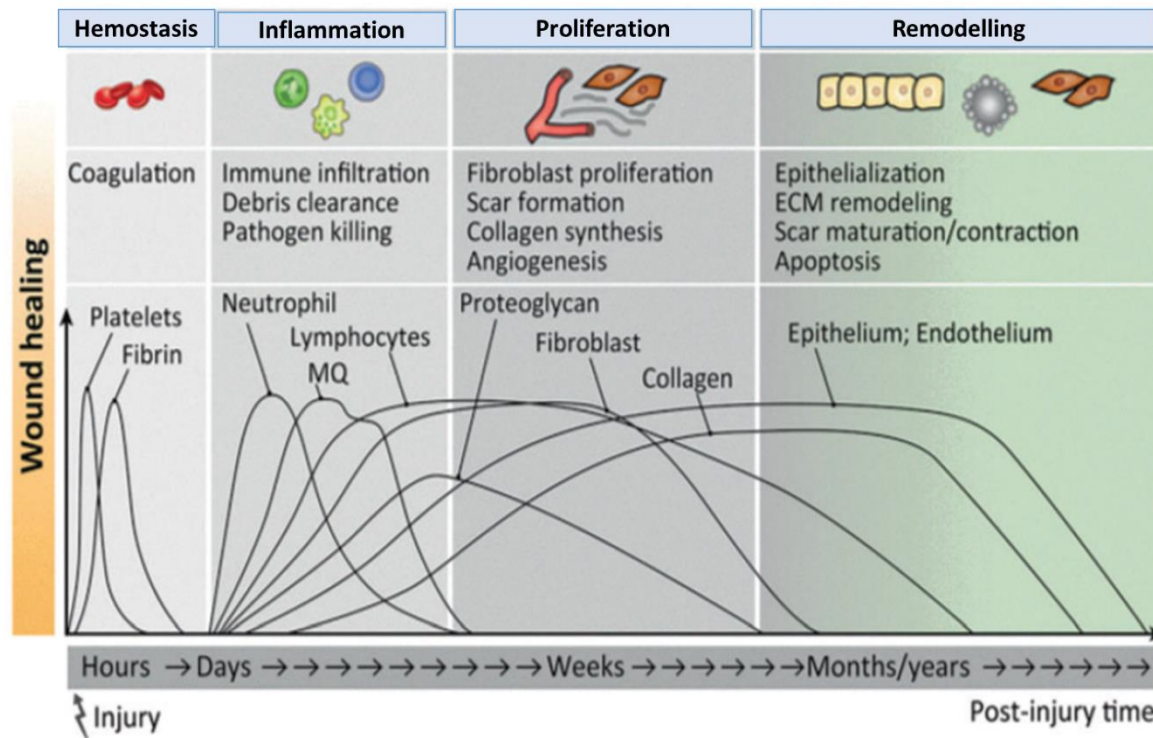


Figure 1.3 Wound healing model time line. The four phases of the wound healing process are illustrated: hemostasis; inflammation; proliferation; and remodelling. Keratinocytes are important in the remodelling phase during epithelialization. Image sourced from Mele [52].

While the role of epidermal keratinocytes in the protection against injury was valued for a great period of time, the role they play in immunological defence only attracted attention many years later, when it became known and appreciated, that keratinocytes produce abundance of growth factors, cytokines, as well as chemo-attractants [53]. Furthermore, it has been demonstrated that in skin-related diseases, such atopic dermatitis and psoriasis, where keratinocytes and lymphocytes are involved, there is crucial signalling between these two types of cells [54]. Their importance in immunology is further demonstrated in conditions such as inflammatory reactions and wound healing, during which they respond to injury in the epidermis by becoming activated. In this activated state, they begin to produce and respond to growth factors and cytokines, they become migratory, and gain the ability to produce basement membrane constituents [55]. Once activated, keratinocytes will express a specific set of keratin proteins that is different to the keratins expressed in the healthy epidermal layer. Activated keratinocytes in the suprabasal layer, will not express K1 and K10 as described above, but will instead produce K6 and K16 [56, 57]. K17 keratin has also been identified when inflammatory processes occur during psoriasis, which involves the infiltration of the Th1 lymphocytes [54, 58]. Activated keratinocytes begin to produce growth factors for signalling, as well as cytokines such as IL-3, IL-6, and IL-8 [59, 60], which are chemotactic attractants for white blood cells

and paracrine (acting upon other cells) for fibroblasts, lymphocytes, and endothelial cells [61]. The signals are also autocrine (acting upon itself) for the keratinocytes.

It has also been shown, that through cytokines that are either Th1- or Th2- lymphocyte derived, are capable of differently regulating gene expression of keratin in the epidermis [53, 54]. While keratinocytes produces and secrete its own set of cytokines, cytokines that are produced by other neighbouring epidermal or infiltrating cells, can affect and influence the production of keratinocyte-derived cytokines, either directly or indirectly. IL-4, IL-13 and IL-17 are a few of these cytokines [62-64]. Keratinocytes express the IL-4 receptor, at mRNA and protein level. It has also been demonstrated that treatment with IL-4 promotes the proliferation of keratinocytes, in addition to the production of keratinocyte-derived IL-6 [62, 63, 65]. Furthermore, the IL-13 receptor has also been illustrated on keratinocytes, and as with the treatment with IL-4, treatment with IL-13 increases the production of keratinocyte-derived IL-6 [64, 66]. In addition to the IL-4 receptor, receptors for IL-17 are also present, and when activated, it mediates the effect of IFN- γ or IL-4 on the process of keratinocyte activation [62, 67].

The use of mouse models have been extremely valuable in providing information about the functions and systemic effects with respect to cytokines and their receptors, and keratinocytes [68-70]. Using transgenic mice has improved our knowledge on the role of cytokines with respect to the epidermis, and additionally, specific keratin promoters, such as the K14 promoter (or KRT14^{cre}), has provided us with vital information regarding the expression of cytokines targeted to the epidermal layer [71-75].

1.2 IL-4/IL-13

1.2.1 IL-4

As described above, IL-4 is extremely important for the establishment and regulation of a Th2 immune response. The genes coding for the IL-4 cytokine in mice (14-19 kDa glycoprotein) are closely situated to the genes coding for IL-5, IL-13 and the granulocyte macrophage-colony stimulating factor (GM-CSF) [76]. IL-4 is secreted by numerous cell types including: conventional and $\gamma\delta$ T cells [77-79]; mast cells [80]; basophils [81]; eosinophils [82], and natural killer T (NKT) cells [83], among others. IL-4 is required for growth and survival of T cells [1]. In B cells, IL-4 is crucial for their activation by influencing the expression of low affinity IgE receptor [84], as well as upregulating the expression of the activation markers MHCII, CD80 and CD86 [85], for promoting growth and class-switching to IgG1 and IgE in mice and suppressing the production of the IgM, IgG2a and IgG2b antibody classes [86, 87].

In mast cells, IL-4 is required for growth [1]. IL-4 is also essential in host protective responses elicited against helminth infections [88-91], as well as in the pathogenesis of asthma [92, 93] (Figure 1.4). While it is generally accepted that IL-4 is central for Th2 immunity, certain studies using mouse models, have proved otherwise, illustrating that Th2 immune responses can be independent of IL-4 signalling [94-97].

1.2.2 IL-3

The IL-13 gene which codes for a 10-14 kDa immunoregulatory protein, is situated on chromosome 11, between the genes coding for IL-4 and GM-CSF genes [98, 99], with a few biological functions shared between IL-13 and IL-4 [100]. IL-13 is produced by various cell types, including T cells, DCs, mast cells, basophils, and NK cells [101-103]. IL-13 is essential in regulating immune responses against asthma (Figure 1.4), allergy and anaphylaxis [92, 93]. Additionally, IL-13 effector functions influences various cells including macrophages, B cells, eosinophils, fibroblasts, mast cells, and smooth muscle cells [104]. Unlike IL-4, IL-13 cannot act on T cells [105]. On monocytes and macrophages, IL-13 upregulates the expression of integrins such as CD11b and CD11c [105]. The IL-13, like IL-4, is important for proliferation and activation of B cells [106].

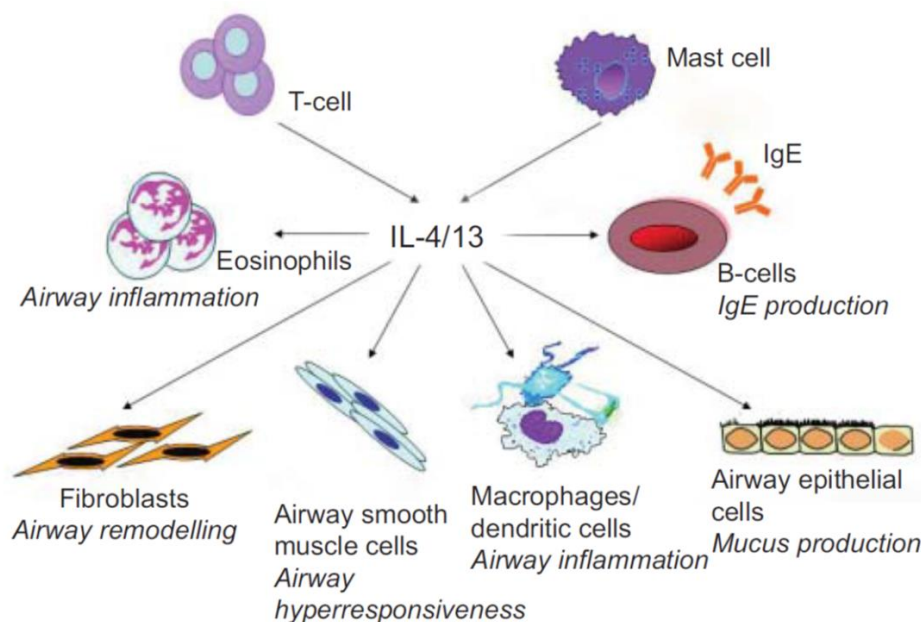


Figure 1.4 Schematic diagram of the potential cellular effects of IL-4/IL-13 on inflammatory and structural cells in asthma. Th2- mast cell-derived IL-4 and IL-13 promotes airway inflammation via the activation of eosinophils, macrophages, and dendritic cells; airway remodelling by enhancing the proliferation and activation of fibroblasts; IgE production by activating B cells; mucus production by stimulating airway epithelial cells and goblet cells; and airway hyper-responsiveness by activating airway smooth muscle cells. Image sourced from Oh et al. [107].

1.2.3 IL-4 and IL-13 receptor complexes

IL-4 and IL-13 share a common receptor, the IL-4 receptor alpha chain (IL-4R α), which may contribute to similar functions shared between the two cytokines [11, 94, 108]. In mice, the IL-4R α is 785 amino acids in length, with a long cytoplasmic region consisting of 5 conserved tyrosine amino acid residues, suggesting that tyrosine phosphorylation may be important in IL-4R α function [109]. The IL-4R α belongs to the hematopoietin receptor superfamily, a family important for the signalling of various cytokines as well as growth factors on hematopoietic cells [108]. Apart from being expressed on hematopoietic cells, IL-4R α is also expressed on endothelial and epithelial cells, muscle cells, brain tissue, etc. [108].

IL-4 signals exclusively through the heterodimer type 1 receptor, which is composed of the IL-4R α subunit and the common gamma chain (γ c) [108, 110], with both IL-4 and IL-13 signalling through the type II receptor, that is composed of the IL-4R α and IL-13-binding receptor chain (IL-13R α 1) subunits [108, 110]. The receptors through which IL-4 and IL-13 signalling is mediated, is illustrated in Figure 1.5 [107]. Binding of the cytokines to the receptors varies. Alone, IL-13R α 1 is bound specifically by IL-13 with low affinity, however, when the IL-13R α 1 is combined with IL-4R α as a unit, IL-13 binds to this with a high affinity [98, 110, 111]. Furthermore, IL-13 will bind with a high affinity to the homodimeric IL-13R α 2, which for a long time was regarded as a decoy receptor fulfilling no signalling requirements, mostly due to the cytoplasmic domain being fairly short with no binding motifs for signalling [112]. Recent work has, however, demonstrated otherwise, providing evidence that IL-13R α 2 is indeed responsible for the induction of the TGF- β production and the mediation fibrosis in Crohn's disease, during chronic disease [113-115]. These IL-13 receptors are found to be expressed in both haematopoietic cells (except T cells) and non-haematopoietic cells in mice and humans [98, 116, 117]. IL-4 and IL-13 may bind with a high affinity to soluble forms of IL-4R α and IL-13R α 2, which cannot signal, and in doing so, this allows the receptors to competitively inhibit the two cytokines, consequently regulating the activity of the IL-4 and IL-3 [118, 119].

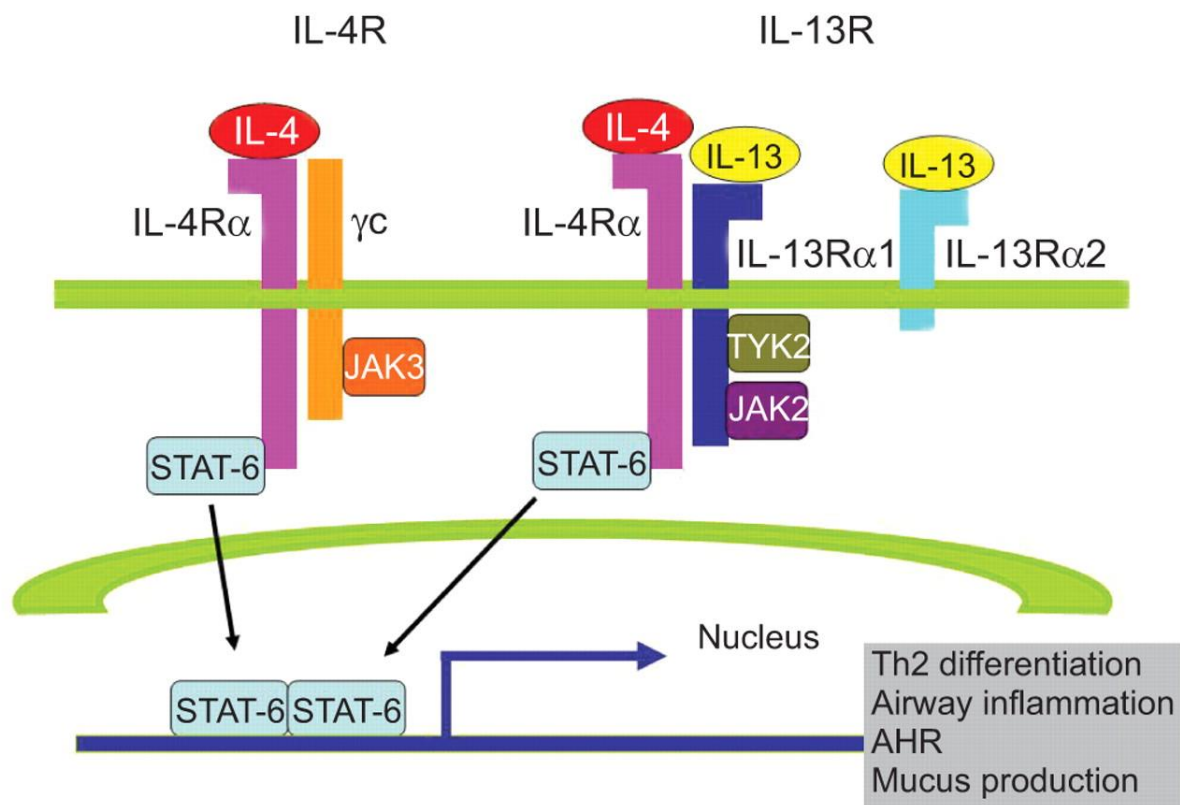


Figure 1.5 Schematic diagram of the IL-4/IL-13/STAT-6 signalling pathways. IL-4 and IL-13 signal via the IL-4R α , a component of the type I (IL-4R α and γ c) and type II receptors (IL-4R α and IL-13R α 1). IL-4 signals via both type I and II receptor pathways, while IL-13 signals only via the type II IL-4R. IL-13 also binds to the IL-13R α 2 chain, which does not contain a transmembrane signalling domain and was thought to act as a decoy receptor. γ c activates JAK3, while IL-13R α 1 activates tyrosine kinase 2 (TYK2) and JAK2. Activated JAKs will then phosphorylate STAT-6. Phosphorylated STAT-6 dimerises, and migrates to the nucleus, to binds to the promoters of the IL-4 and IL-13 responsive genes associated with Th2 cell differentiation, airway inflammation, airway hyper-responsiveness and mucus production. Image sourced from Oh et al. [107].

1.2.4 Mechanisms of IL-4 and IL-13 signalling through the IL-4R α chain

Signalling of IL-4 and IL-13 through the IL-4R α , leads to the activation of the STAT-JAK signalling pathway [108]. IL-4 binds to IL-4R α which induces the activation of JAK proteins, and results in tyrosine phosphorylation of the IL-4R α chain. STAT-6 then binds to the phosphorylated receptor, allowing for C-terminal domain phosphorylation by the activated kinases. Thereafter, the activated STAT-6 is released from the bond with the receptor, and via interaction of the phosphorylated C-terminal domain with a conserved domain, forms homodimer components. These STAT-6 homodimers then migrate to the nucleus, to bind to specific DNA motifs present in the promoter of responsive genes, resulting in enhanced transcription. Signalling of IL-4 via IL-4R α has also been shown to trigger the

phosphoinositide-3-kinase pathway and insulin receptor substrate signalling pathway [107, 120-125].

1.2.5 Cell-specific IL-4R α

The use of gene-deficient mice has been extremely useful in delineating the role of the IL-4R α signalling molecule in protection or susceptibility of hosts to pathogen infections. Advances in genetics have, however, introduced a more advanced method, i.e. the Cre/*loxP* recombination system, allowing for the deletion of a gene on a specific cell-type [126-129]. In this system, a mouse that has been genetically modified, i.e. a transgenic mouse, carries a Cyclization recombinase (Cre) gene, which is under the control of a promoter that is specific for a particular cell-type. This transgenic mouse is mated with another transgenic mouse that has been modified to carry the gene of interest, and is flanked by a pair of *loxP* binding sites. Upon mating, an inter-crossing of genes occurs, and the promoter will induce the expression of the Cre, but only on those specific cell-types, and the enzyme carries the gene deletion via homologous recombination of the *loxP* sites. In order to achieve a deletion of IL-4R α on a specific cell type, our laboratory generated a transgenic mouse that carries IL-4R α gene exon 7 to exon 9 that is flanked by *loxP* sites. Several cell specific-IL-4R α knock-out mice have been generated in our laboratory. These cells include pan T cells [130], CD4⁺ T cells [131, 132]; B cells [133], macrophages [134], dendritic cells [135] neutrophils [134], and smooth muscle cells [136, 137].

1.3 Leishmaniasis

1.3.1 Background

Leishmaniasis is a neglected tropical disease [138, 139], and is regarded as the third most important vector-borne disease, after malaria and African trypanosomiasis. Globally, leishmaniasis is ranked at 9th place, with respect to the burden of disease of all infectious as well as parasitic diseases [140]. The causative agent, the *Leishmania* parasite, is an obligate, intracellular microorganism that belongs to the trypanosomatida protozoan genus [138, 139, 141, 142]. It is estimated that approximately 350 million people are at risk of contracting leishmaniasis [143, 144] which is endemic in 98 countries, occurring over five continents [143, 145]. Each year, roughly 900 000 to 1.3 million cases arise, with 20 000 to 30 000 deaths occurring [139, 141, 145]. Furthermore, leishmaniasis is associated with approximately 2.4 million disability-adjusted life years [144, 146, 147].

Leishmaniasis occurs when *Leishmania* parasites are transmitted to a vertebrate host by the bite of a female Phlebotomine sandfly (2 to 3 mm insects) [139, 142, 148]. Approximately 70 species of animal hosts commonly infected include humans, cats and dogs. There are over 20 species of *Leishmania* parasites that cause the disease, and over 90 species of sandflies responsible for transmission [139, 142, 148]. In humans, the clinical forms of the leishmaniasis may be classified into one of three groups: visceral leishmaniasis (VL) (also known as *kala-azar*), which is the most lethal form; cutaneous leishmaniasis (CL), the most common form; and muco-cutaneous leishmaniasis (also known as *espundia*) [139-142].

Visceral Leishmaniasis may be caused by one of the following parasites: *L. donovani*; *L. infantum*; *L. chagasi*; or *L. amazonensis*. If left untreated, VL is fatal in most cases (over 95%). Symptoms of VL include irregular episodes of fever, night sweats, enlargement of the liver and spleen, anaemia, weakness, anorexia, and weight loss [139, 144, 149, 150]. Diagnosis of VL includes serological diagnosis e.g. ELISA, and parasitological diagnosis e.g. using aspirates of the spleen, bone marrow or lymph nodes [139, 144, 151, 152]. Every year, approximately 200 000 to 400 000 new cases of VL occurs globally. Visceral leishmaniasis is endemic in East Africa and the Indian subcontinent. The species of *Leishmania* parasite responsible for visceral leishmaniasis varies with location: *L. donovani* in the Africa, Asia, and the Indian subcontinent; *L. infantum* or *L. chagasi* in southwest and central Asia the Mediterranean region, and South America (primarily in young children); *L. amazonensis* in South America; and *L. tropica* in the Middle East [139, 146, 147, 153]. A sequel of VL, Post-kala-azar dermal leishmaniasis (PKDL), also exists. This manifests as a nodular rash on the face upper arms, and other parts of the body [139, 152-154]. Treatment of VL includes the use of the first-line drugs pentavalent antimonials, as well as the second line drugs amphotericin B and amphotericin B in liposomes [76, 139, 144, 152, 155].

Cutaneous leishmaniasis presents as ulcerations on the dermis or skin, and is generally localized to tissue that surrounds the inoculation site, however, during a chronic type infection, the parasites can migrate to the draining lymph nodes [139, 140, 144, 156], and roughly 90% of all cases occurs in Afghanistan, Pakistan, Syria, Saudi Arabia, Algeria, Islamic Republic of Iran, Brazil, and Peru [147, 157]. CL may be caused by various parasite species, depending on location. Old world CL (Africa, Asia and Europe) is caused mostly by *L. major*, *L. tropica*, and *L. aethiopica*, Mediterranean and Caspian sea regions CL includes *L. infantum* and *L. chagasi*, and New world CL (America) includes *L. mexicana*, *L. amazonensis*, *L. braziliensis*, *L. panamensis*, *L. peruviana*, and *L. guyanensis*. Each year approximately 0.7-1.2 million new cases of CL arise [139, 147]. Figure 1.6 illustrates the distribution of reported cases worldwide

in the year 2013 [158]. Diagnosis of CL includes parasitological diagnosis based on skin smears, and PCR [159-161]. While old world CL lesions are primarily papules, nodules, or nodule-ulcers, new world CL presents primarily as ulcerative lesions [144, 160, 162]. CL treatment involves the administering of pentavalent antimonials (local for few, small lesions and systemically for several, large lesions) [159, 161, 163].

Mucosal dissemination of cutaneous leishmaniasis or muco-cutaneous leishmaniasis (caused by *L. braziliensis*, *L. panamensis*, *L. guyanensis*), may occur in 1-10% of infections [139, 164]. Diagnosis of muco-cutaneous Leishmaniasis uses serological techniques due to the strong humoral response these patients tend to develop [139, 144, 165]. This form of leishmaniasis may develop during cutaneous leishmaniasis, or between 1 and 5 years after cutaneous leishmaniasis heals [144, 164]. Muco-cutaneous leishmaniasis presents as destructions of the mucous membranes of the nose, mouth and the throat, starting with ulcerations at the nares, followed by perforation of the nasal septum as well as the formation of destructive inflammatory lesions, ultimately causing obstructions in the pharynx or larynx and consequently causing disfigurement [139, 166]. Muco-cutaneous treatment includes the administering of pentavalent antimonials as above [144, 167].

Control strategies to curb spread of disease includes vector control, and case identification and treatment. There have been several approaches made in the development of a vaccine for Leishmaniasis, however, to date, none of these have been successful in humans [168, 169]. Furthermore, a significant increase in the number of cases has been observed in various parts of the world, and may be attributed to different factors, some of which include the following: environmental changes that increase exposure to the sandfly vectors; large-scale migrations of populations for occupational reasons (border crossing); deterioration in the social and economic conditions in especially low income populations; and the rise in leishmaniasis-HIV infections [139, 146, 170-173]. For these reasons, efforts to further understand the immune responses against leishmaniasis, to identify vaccine or therapeutic drug targets, is becoming increasingly important.

Status of endemicity of cutaneous leishmaniasis, worldwide, 2013

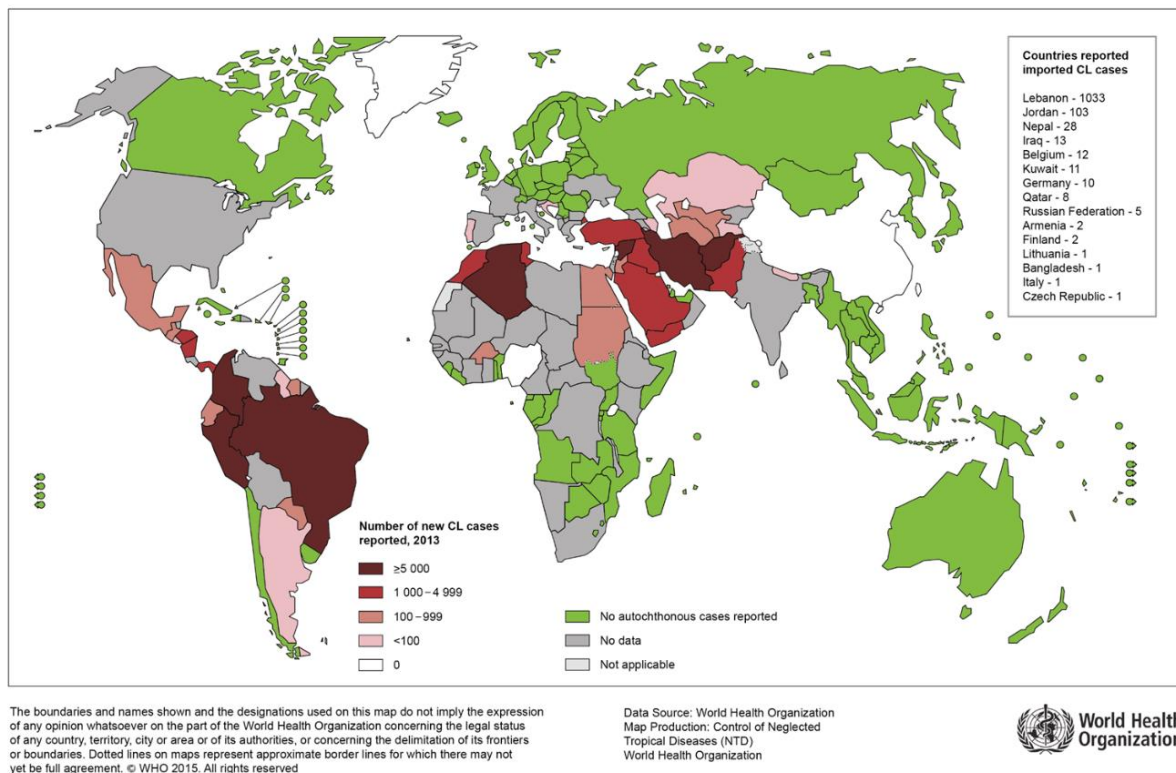


Figure 1.6 Global distribution of cutaneous leishmaniasis in 2013. Image sourced from WHO [158].

1.3.2 Life cycle of *L. major*

Figure 1.7 demonstrates the life cycle of the *Leishmania* parasite in the sandfly vector and human host. Briefly, a *Leishmania* infected female sandfly takes a blood meal, during which she regurgitates and introduces metacyclic promastigote parasites, along with immunomodulatory parasite-derived proteophosphoglycans and salivary factors into the dermis of the human host. Numerous cells are recruited to the site of inoculation, including macrophages, which phagocytose the promastigotes. These promastigotes, while internalised in the cells, transform into aflagellate amastigote parasites. Amastigotes multiply within the cells, followed by cell rupturing to release the parasites, and allowing for the infection of surrounding phagocytic cells. The cycle continues with the next blood meal feed, when these infected cells are taken up by a female sandfly. The amastigotes next transform into promastigotes in the midgut, followed by differentiation of procyclic promastigotes into infective metacyclic promastigotes. These parasites reside in the midgut, awaiting transmission during a blood meal [174, 175].

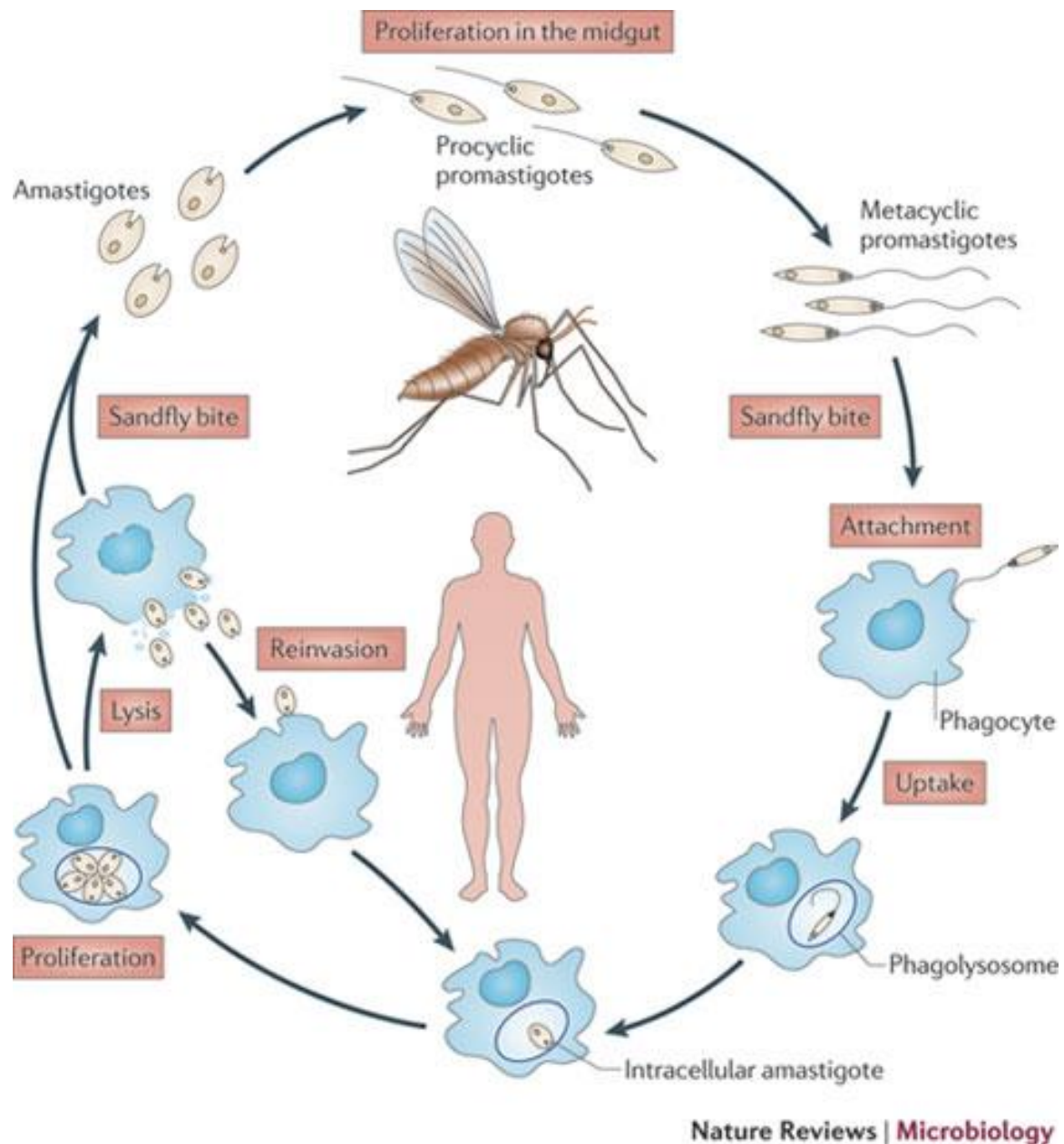


Figure 1.7 Life cycle of *Leishmania* parasite. The life cycle begins in the midgut of the female sandfly, with the differentiation of *Leishmania* procyclic promastigotes into infective and non-dividing metacyclic promastigotes. During her blood feed, the sandfly regurgitates metacyclic promastigotes, along with salivary components and immunomodulatory parasite-derived proteo-phosphoglycans. The metacyclic promastigotes are next phagocytosed by one of cell types recruited to the infection site, and thereafter, metacyclic promastigotes transform into aflagellate amastigotes. The amastigotes undergo replication within the host cells, when they become too many, the cells burst to release infective amastigotes, ready to infect neighbouring cells. During a blood feed, the sandfly will take up infected phagocytes, and the contained amastigotes will transform into promastigotes in the midgut, completing the transmission cycle [174]. Image sourced from Kaye *et al.* [174].

1.3.3 The immune response to *L. major*

The immune response to *L. major*, more specifically, resistance and susceptibility to the parasite, has been extensively investigated, with the use of experimental mouse models. The

possible outcome of *L. major* infection, resistance or susceptibility, is dependent on the cellular and humoral responses. CD4⁺ T helper cells play a major role with a polarised Th1 immune response, characterised by the predominant production of IFN- γ , IL-12 and TNF- α , linked to resistance; and a polarised Th2 response, characterised by the predominant production of IL-4, IL-13, IL-10 and TGF- β associated with susceptibility [175-182].

The first cells to migrate to the infected sites are the neutrophils. Here, they release antimicrobial mediators, and engulf microorganisms, to phagocytose them. Furthermore, neutrophils are responsible for killing foreign infectious by creating a potent oxidative burst to release toxic mediators [183, 184]. Neutrophils may kill *Leishmania* parasites or protect them, based on the species of parasite as well as the infected host [185]. While *L. amazonensis* promastigotes are killed by neutrophil extracellular traps (NETs) [186, 187], it has been demonstrated that salivary proteins transferred from the sand fly during the blood feed has the ability to protect the parasites against neutrophil-mediated death [188]. For this reason, it is still unclear whether NETs play a role in protection. In the case of *L. braziliensis* and *L. amazonensis*, neutrophils aid in controlling infection by interacting with infected macrophages [189, 190]. Conversely, the uptake of apoptotic neutrophil cells by macrophage and dendritic cells, after infection with *L. major*, has shown to decrease the activation of the macrophages and dendritic cells. This allows the parasite to survive for longer periods, and evade the host immune responses [191, 192]. Neutrophils are able to enhance their chances of uptake by dendritic cells, due to their increased expression of apoptotic markers, as well as increased CC-chemokine ligand 3 (CCL3)-dependent recruitment of dendritic cells [193]. Decreased activation of dendritic cells, results in reduced Th1 response. Additionally, this inhibits cross-presentation for CD8⁺ T cell activation [194, 195]. Murine *L. major* models have shown that the role of neutrophils in the immune response is dependent on the genetic background of the infected host infection. In susceptible BALB/c mice, depletion of neutrophil cells was shown to inhibit the early IL-4 response, which prevented Th2 driven non-healing response to occur. In C57BL/6, however, the depletion of neutrophils had no effect on the course of infection [196].

Inflammatory monocytes and dendritic cells recruited to the site of infection, are the cells predominantly infected with *Leishmania* parasites, during the first few days after infection parasites [194]. The recruitment of inflammatory monocytes depends on the production of CCL2 by cells at infection site, after activation via the platelet-derived growth factor [197]. Unlike monocyte cells which display a strong respiratory burst after infection, and results in

early parasite control, the macrophages require activation by IFN- γ for eradication of the parasites [197]. Essentially, inflammatory monocytes are necessary for the control of infection.

The complement protein and opsonin (antibody or substance that binds to foreign micro-organisms or cells to render them more susceptible to phagocytosis), C3b, binds to *L. major*, allowing for macrophage cells to phagocytose them. The C3b is, however, converted to iC3b, by a glycoprotein found on the surface of the *Leishmania* parasite, gp63, and inactivated in the process [198]. Macrophages respond to infection by producing superoxide and hydroxide radicals against the parasites, while *L. major* parasites inhibit this reaction by producing acid phosphatases on its surface. Macrophages produce acidic enzymes in an attempt to degrade the parasites [199, 200], when lysosomes fuse with the phagosome. *L. major* circumvents this through the use of a proton pump on the surface of the parasite, allowing them to maintain their pH at a neutral position. *Leishmania* Lipophosphoglycan (LPG) is also important for the inhibition of the enzymes. The longer the parasites reside in the macrophage cells, the longer they evade the immune system [201].

Reactive oxygen species (ROS) is produced by the respiratory burst that follows uptake during the phagocytic process. Nitric oxide (NO), is produced by inducible NO synthase (iNOS) that occurs after cells are activated by IFN- γ . These two products are crucial in *L. major* parasite control. *Leishmania* parasites are sensitive to ROS, however, respiratory burst in non-activated macrophages after infection is not sufficient in killing the parasites [202]. This may be resultant of the ability of parasites to inhibit the generation of ROS in phagolysosomes [203]. IFN- γ does, however, improve respiratory burst in the macrophage cells, which allows for better parasite clearance [204]. In order for macrophages to be sufficiently and optimally activated for parasite clearance, the induction of iNOS by IFN γ and tumour necrosis factor (TNF) needs to occur [205, 206]. NO diffuses across the cell membrane, and can mediate killing of intracellular parasites contained within the NO-producing cell, as well as parasites contained in neighbouring cells [207]. While mouse models have demonstrated that NO is crucial in *Leishmania* parasite control [208], their role in humans remains unclear.

In mice, IL-12 is important in the development of the CD4⁺ Th1 cells, and a protective response [209, 210], while the predominant secretion of IL-4 leads to the promotion of the development of CD4⁺ Th2 cells and susceptibility [211]. Dendritic cells are the primary source of IL-12, and initiates antigen-specific immunity to *Leishmania* [212]. While some dendritic cells that prime the naive T cells reside in the draining lymph node [213], majority results from the recruitment

of inflammatory monocytes to the cutaneous lesion, which differentiate into monocyte-derived dendritic cells that travel to the lymph node [214].

Prior to Th1 cell development, the natural killer (NK) present in the draining lymph node (and are closely situated to the dendritic cells) are the main source of IFN- γ [215]. After NK cells are activated, they are recruited to the paracortex and produce IFN- γ [216], promoting IL-12 production by DCs. Regulation of the NK cell response is brought about by the production of Transforming growth factor β (TGF β), which reduces the production of IFN- γ [217]. CD8+ T-derived IFN- γ may also drive the early adaptive immune response to *L. major*, but this is dependent on the extent of the initial infection [218, 219].

As explained above, CD4⁺ Th1 cells are needed to control *L. major* infections. Following infection, CD4⁺ Th1 cells are recruited to the lesions, and produce IFN- γ which activates macrophages for parasite clearance. In humans suffering from cutaneous leishmaniasis, an additional cell population, is expanded and present. These are identified as double-negative T cells, and do not express CD4 or CD8, however, do express CD3 and $\alpha\beta$ TCR. Similarly, in *L. major*-mouse studies, a double-negative T cell population has been identified. While these cells may be phenotypically different to the classical CD4⁺ T cells, exhibiting an innate cell-like gene expression profile, they proliferate, as well as secrete IFN- γ upon MHCII antigen recognition of *L. major*, similar to classical CD4⁺ T cells, and therefore do contribute to immunity [220, 221].

Despite clearance and resolution of infection, some parasites may remain as a result of the downregulation of the immune response by IL-10 [222], and these persistent parasites retain a population of short-lived *Leishmania*-specific T cells, the effector CD4⁺ T cells, which may respond immediately in the event of re-infection. Additionally, there are short-lived *Leishmania*-specific T cells with an effector memory T cell, and long-lived central memory T cells [223]. CD4⁺ central memory T cells travel to the draining lymph node, proliferating and differentiating into effector T cells, which then travel to the site of the lesion [223, 224].

The role of Treg cells in humans is still unclear, in contrast to their role in mice [225]. IL-10 is an important regulator of immunity in murine Leishmaniasis. During infection with *L. amazonensis* and *L. panamensis*, in mice that received Treg cells from an infected mouse prior to infection, pathological responses were reduced, and disease progression halted, evident in decreased lesion sizes and lower parasite burdens, and reduces lesion development. This demonstrated that Treg cells may control immunopathological responses [225, 226]. During *L.*

major infection, mice lacking IL-10 have been shown to control the replication of parasites [227, 228]. Sources of IL-10 include Th1 cells, Treg cells, B cells [229] and macrophages that have been exposed to IgG-coated *L. major* amastigotes [227].

B cells also have the ability to stimulate CD4⁺ T cells [230, 231]. Recent studies have demonstrated that B cells are required for susceptibility to infection with *L. major* strain LV39 in BALB/c mice, and were shown to play an important role as APCs, instructing the development of a Th2 polarised response [232]. In response to a Th1 polarised response, B cells will secrete the Type 1 antibodies IgG2a, IgG2b and IgG3, while stimulation of B cells by Th2 characteristic IL-4, leads to isotype switching to produce Type 2 IgG1 and IgE antibodies [233].

1.3.4 IL-4/IL-4R α signalling in cutaneous Leishmaniasis

Experimental infections in mice have been extremely useful in gaining insight on the roles of IL-4 and IL-4R α in *L. major* infection. More specifically, the use of cell-specific IL-4 and IL-4R α knock-outs as described above, have been instrumental in providing information on the role for this cytokine/receptor pair in *L. major* infection. Early mouse studies demonstrated that generally susceptible BALB/c mice that lacked IL-4 demonstrated a resistant phenotype when infected with *L. major*, and were able to control the progression of disease [179]. During the acute phase of *L. major* infection in IL-4^{-/-} and IL-4R α ^{-/-} deficient BALB/c mice, animals appeared to control the infection, evident in reduced swelling and parasite burdens, with predominant Th1 and Type 1 antibody responses. Over a longer period, however, in the chronic stage, IL-4R α ^{-/-} mice appeared to be highly susceptible, seen in increased disease progression, with increased swelling and necrosis of the lesions on the footpad [233]. Abrogation of IL-4 signalling on CD4⁺ T cells was shown to be required to transform susceptible non-healing BALB/c mice to a resistant healing phenotype, which was evident in the decreased swelling and low parasite burdens [132]. A study to ascertain whether the signalling of IL-4R α on macrophage cells in BALB/c mice (LysM^{cre}IL-4R^{-flox} BALB/c) is required for protection during *L. major* infection, demonstrated that the mice lacking the receptor on these cells, had delayed progression of disease, predominant type 2 Ab responses, and significantly increased levels of IFN- γ . These authors demonstrated that alternatively activated macrophages play a role in the susceptible phenotype in non-healing BALB/c mice [234]. A study of note is the work carried out by Biedermann *et al.*, who demonstrated the principle of the “IL-4 instruction theory” [235]. Here they showed that IL-4 can instruct dendritic cells to drive a Th1 polarised immune response, evident in *in vitro* experiments where the production of IL-12 and IFN- γ

was increased, on the addition of IL-4 to bone marrow-derived dendritic cells. *In vivo* mouse experiments, demonstrated decreased swelling and lesion sizes, when mice were administered recombinant IL-4 within the first 8 hours of infection, supporting a Th1 resistant phenotype as above. Conversely, when IL-4 was administered after 12 hours, IL-4 was upregulated and IFN- γ downregulated. This suggested that early IL-4 was required to drive a Th1 response. More recently, dendritic cell-specific IL-4R α BALB/c mice infected with *L. major*, showed hyper susceptibility to infection, seen in significantly higher swelling and parasite burdens, as well as an upregulation of Th2 cytokines and type 2 antibodies. This indicated that IL-4R α -responsive dendritic cells are crucial to the hosts survival [135], and further supported the findings reported by Biedermann *et al.* [235].

1.3.5 A role for keratinocytes

During the blood-feed, the sand fly causes significant damage to skin, which results in the rupturing of the dermis and capillaries within. This creates a blood “pool” which contains extracellular matrix (ECM) constituents from the skin tissue and blood, including a variety of cells [236, 237]. This occurrence attracts the neutrophils [237], and macrophage cells, which are the primary host cell type for the *Leishmania* parasites [238]. This interaction facilitated between promastigotes, the ECM, and the immune cells is crucial in the establishment of the infection, as well as the immune response elicited. Given the situation of keratinocytes in the epidermis, these cells are bound to play some role in the immune response against *L. major*. The presence of *L. major* promastigotes has been shown to rapidly induce keratinocytes to secrete immunomodulatory mediators such as IL-12, IL-1 β , IL-4, and IL-6, in a study using laser-microdissection and in-situ-hybridization. Notably, the secretion of IL-6 by these cells was found to be higher in genetically resistant mice, when compared to the levels in susceptible ones, and mice with a selective deficiency for IL-6 in non-hematopoietic cells demonstrated a non-healing phenotype. In addition, the secretion of the Th2 cytokine IL-4 from keratinocytes, as determined by laser microdissection and PCR techniques, was also significantly highly present in surprisingly, resistant C57BL/6 mice and to a lesser extent in BALB/c mice. Early local neutralization of the IL-4 in these resistant mice resulted in a Th2 switch, rendering these mice susceptible [239]. This further supports the suggestion that keratinocytes play a crucial role in immune response elicited against *Leishmania*. Considering the findings that early IL-4 instructs dendritic cells to drive a Th1 response [235], and that keratinocytes at the infected site have been shown to have upregulated IL-4 in resistant mice [239], we hypothesize that the

early IL-4 responsible for the Th1 polarisation, is derived from keratinocytes at the site of infection.

1.4 Schistosomiasis

1.4.1 Background

Schistosomiasis is a neglected tropical parasite disease (acute and chronic) caused by blood flukes, the trematode flatworms, belonging to the *Schistosoma* genus. It is also referred to as bilharzia and snail fever. Of the 72 countries in which schistosomiasis currently occurs in, it is endemic in 52 of them [240, 241]. The global distribution of schistosomiasis in 2011 is illustrated in Figure 1.8 [242]. Each year, schistosomiasis affects approximately 200 million people, killing more than 100,000 individuals, with the greatest number of cases occurring in Africa, the Eastern Mediterranean and the Americas. The causative agents of Schistosomiasis in humans includes *S. mansoni*, *S. japonicum*, *S. haematobium*, and *S. intercalatum*. Our study focuses on *S. mansoni*, which occurs primarily in Africa, the Middle East and Latin America [241, 243-245]. *Schistosomiasis* is a major public health concern in some countries, due to aspects that favour transmission of the disease in endemic areas, including poor socioeconomic conditions, the use of contaminated water, difficulties in access to health services, and inadequate water and sewage treatment [244, 246, 247]. The symptoms experienced during schistosomiasis are a result of the reaction of the hosts' body to the *Schistosoma* worm eggs. These include abdominal pain, diarrhoea, bloody stools, and the enlargement of spleen and liver organs, among others. [241, 244]. Diagnosis may include physical examination to perform abdominal palpation, to look for hepatomegaly and/or splenomegaly. The gold standard test for diagnosis of schistosomiasis is, however, microscopic examination of excretory remains (stool, urine) [248]. The control against all forms of schistosomiasis is primarily preventive chemotherapy using the trematocide Praziquantel, which is effective and safe, as well as fairly affordable [241, 244, 248, 249]. There have been over 100 schistosome vaccine antigens that have been identified, however, only a quarter of these have displayed some level of protection when tested using murine models of schistosomiasis. Unfortunately, only three molecules, *S. mansoni* fatty acid binding protein (Sm14), *S. mansoni* tetraspanin (Sm-TSP-2) and *S. haematobium* glutathione S-transferase (Sh28GST), have progressed to human clinical trials, while a fourth, Smp80 (calpain), is at the non-human primate stage of testing [250-252]. Due to high mortality and morbidity, the fact that *Schistosoma* infections appear to increase the risk of HIV [253-255], and the concerns about co-infections with *Salmonella* and

Schistosoma [256], there is a great need for new drug and vaccine targets against schistosomiasis.

Distribution of schistosomiasis, worldwide, 2011

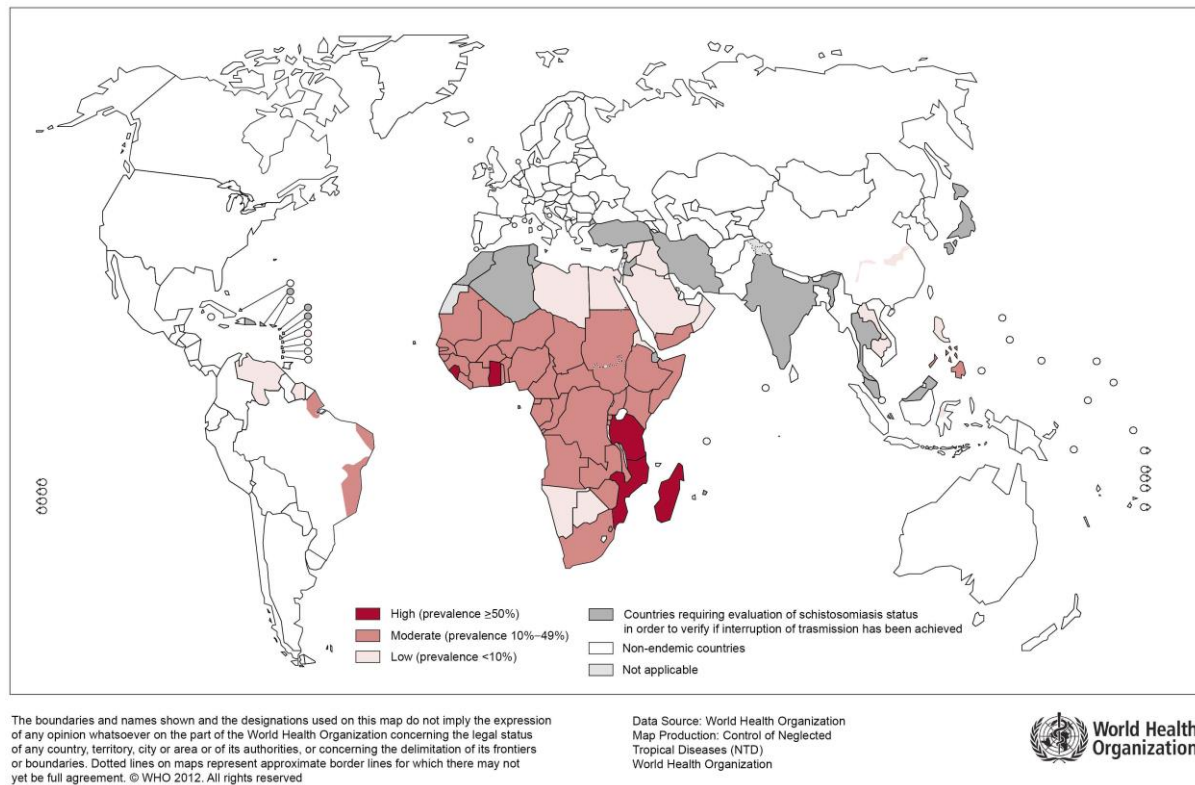


Figure 1.8 Global distribution of schistosomiasis in 2011. Image sourced from WHO [242].

1.4.2 Life cycle of *S. mansoni*

Transmission of schistosomiasis in humans begins when they encounter contaminated water. The cercariae stage of the parasite, freely swims in fresh water, and on contact with human skin, and with the use of proteolytic enzymes, penetrates it. Once inside the human host body, the cercariae transforms into schistosomula, shedding their forked tail, and locates to the vasculature of the host. The larvae migrates to the peripheral vessels, lung and liver, where they mature and eventually mate in the liver vessels to form adult worms. After cercariae penetrating the skin, the schistosomula require approximately 5 to 7 weeks to transform into adult worms. The male and female adult worms reside within the portal veins, where they will eventually mate to produce fertilised eggs which the females lays there. The eggs produce proteases that induce inflammatory reactions which is required for them to be passively transferred through the walls of the intestine and the bladder, to ultimately be eliminated in stool and urine. Some eggs are retained in host, and become trapped in the liver tissues, causing an immune reaction. Here they induce inflammation, form granuloma structures and fibrosis

around them, and eventually die, resulting in organ damage. The eggs, regardless of their fate, will only survive between 1 to 2 weeks after they are released by the female worm. The eggs excreted into freshwater eventually hatch to release the next stage of the parasitic worm, the free-living ciliated miracidia, which is ready to infect a fresh waters mollusc snail host. The snail hosts include the genus *Biomphalaria*, and the species *Biomphalaria glabrata* and *B. tenagophila*, *B. straminea*. In the snail host, the parasite will undergo asexual replication through sporocyst stages and will eventually shed tens of thousands of cercariae, ready to infect a human host. The cercariae have an infective period in freshwater of 1 to 3 days, however, their energy reserves begin to reduce greatly over a few hours. This marks a full cycle. During the lifecycle in the snail, the asexual period requires approximately 4 to 6 weeks before the cercariae are released for human infection [257-260]. The life cycle of the of *S. mansoni*, *S. haematobium*, and *S. japonicum* are illustrated in Figure 1.9 [257].

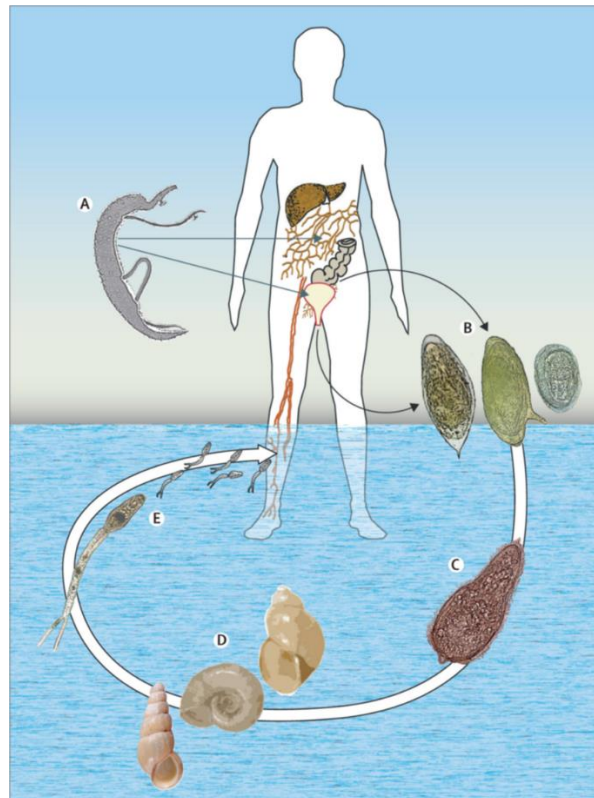


Figure 1.9 The life cycle of *S. mansoni*, *S. haematobium*, and *S. japonicum*. (A) Paired adult male and female worms (larger male enfolding slender female), (B) egg stage (from left to right, *S. haematobium*, *S. mansoni*, *S. japonicum*), (C) ciliated miracidium stage, (D) snail hosts (from left to right, *Oncomelania*, *Biomphalaria*, *Bulinus*), and (E) cercariae stage. Image sourced from Colley *et al.* [257].

1.4.3 The immune response to *S. mansoni*

Murine models have provided extensive insight into the immunology of schistosomiasis. Both cellular and humoral antibody responses are required for optimal protection against schistosomiasis [261]. T cell-deficient mice have indicated crucial roles for immune responses to the maturation of worms, as well as the formation of granuloma structures [262, 263]. The hosts' immune response may be divided into three phases according to the predominant immune response elicited. The first phase occurs 3 to 5 weeks after exposure to parasites, and is characterised by a balanced or dominant Th1 immune response to the parasite antigens [264]. Phase 2 commences around 5 to 6 weeks post-infection, when eggs are produced. During this time, the Th1 response decreases, while a Th2 response becomes the dominant response as a result of the eggs, and the formation of granuloma structures encapsulating the eggs [134, 265, 266]. This immunologic shift is due to the interaction of specific schistosome egg antigens with dendritic cells, and partially through the action of certain carbohydrate epitopes [267]. Th2 immune responses provide a protective function, and sufficient regulation can minimize overall pathology in the host. When Th2 responses are depleted, specifically IL-4, tissue damage and host mortality occurs as a result of pro-inflammatory Th1-type responses [268, 269]. The chronic phase occurs from 10 weeks post-infection. During this phase of the immune response, the Th2 immune response is downregulated with the development of a strong regulatory T cells response, inducing less fibrosis and the formation of granulomas around new eggs which are smaller in size than those produced during the acute infection [270-273]. Other immunomodulatory mechanisms include IL-10 secretion, B cells and antibodies [243, 274].

1.4.4 IL-4/IL-4R α signalling in schistosomiasis

As mentioned above, murine studies have been helpful in better understanding the immunological response elicited against *S. mansoni* infection. The use of genetically modified mice has given us great insight into aspects that contribute to disease outcome during *S. mansoni*. During *S. mansoni* infection, global IL-4 (IL-4^{-/-}) knockout mice appeared to be susceptible to disease, indicating a role for this cytokine in protection [265]. In the absence of both IL-4 and IL-10 (double knockout), mice appeared to be highly susceptible to disease, and demonstrated upregulated levels of Th1 IFN- γ and TNF- α , with increased liver and gut pathology [270]. The IL-4R α /STAT pathway has also been explored for their role in *S. mansoni* disease. Mice lacking IL-4R α ^{-/-} and STAT6^{-/-} demonstrated high susceptibility when infected with *S. mansoni*, evident in the downregulation of Th2 cytokines, inflammation in organ tissues, and reduction in fibrosis [275, 276]. In our laboratory, cell-specific IL-4 and IL-4R α

knockout mice generated using the Cre/loxP recombination system as described above, has been useful in demonstrating roles for these components during *S. mansoni* infection [277]. Various cell types have been explored for the contribution of the IL-4R α found on their surfaces, to *S. mansoni* protection and susceptibility. Mice that lacked IL-4R α signalling on macrophage and neutrophil cells were highly susceptible to *S. mansoni* during the acute stage, and the data indicated that alternative macrophages are required for protection during *S. mansoni* [134]. Studies investigating the signalling of IL-4R α on pan-T cells [130], as well as smooth muscle cells [136], showed that while these mice were able to control inflammation in the gut, they were still susceptible to *S. mansoni* infection, indicating a protective role for IL-4R α on these cells.

1.4.5 A role for keratinocytes

The skin plays an important part in the life cycle of *S. mansoni*. The cercariae need to efficiently and effectively penetrate the skin and migrate through this barrier and its components, while evading any protective mechanisms. The response of keratinocytes to invading cercariae has not been well studied. An early study demonstrated that when mice were infected with *S. mansoni*, the keratinocytes responded by secreting IL-7 [278]. Recently, Bourke *et al.* demonstrated that *S. mansoni* cercariae, as well as their excretory and secretory products may act directly upon epidermal keratinocytes, causing them to respond by initiating barrier repair and pro-inflammatory mechanisms, as observed during epidermal wound healing [279]. Considering the protective role IL-4 and IL-4R α appears to play in *S. mansoni*, the importance of the skin during infection, and the secretion of keratinocyte-derived IL-4 as a Th2 driving factor as suggested by Ehrchen *et al.* during *L. major* infection [239], we postulated that IL-4R α keratinocytes would play a role in protection during *S. mansoni*.

1.5 Objectives of the present study

The purpose of this study was to determine whether IL-4/IL-13 signalling through the IL-4R α on keratinocytes, plays a role in the outcome of disease, using two *in vivo* infection mouse models that require penetration of the skin for establishment of infection.

1. *Leishmania major*
2. *Schistosoma mansoni*

Chapter 2: Deletion of IL-4R α -responsive keratinocytes during cutaneous leishmaniasis causes minor immunological changes in BALB/c and C57BL/6 mice, but does not affect their susceptibility and resistance to infection respectively

2.1 Introduction

The skin is an immune organ, whose primary cell type is keratinocytes [39], and in parasitic infections such as cutaneous leishmaniasis, infective *Leishmania major* (*L. major*) promastigotes are injected into the skin by the female sandfly. The promastigote parasites migrate through this barrier and its components, and establish infection. Due to the abundance of keratinocytes in the epidermis, it would not be surprising if these cells could play immunomodulatory roles during *L. major* infection. Experimental mouse models for *L. major* infections provide a good indication of the immune response elicited against it, which is polarised to either a T helper (Th) 1 or Th2 response, resulting in either resistance or susceptibility to the infection, respectively. While a Th1 response is characterised by classical activation of macrophages via the cytokines interferon gamma (IFN- γ) and interleukin (IL)-12 production, the Th2 response is characterised by alternative activation of macrophages, via the production of various cytokines including IL-13, IL-5, and primarily IL-4, which signals via the IL-4 Receptor alpha chain (IL-4R α). Previous studies have well-demonstrated that a resistant phenotype is observed in C57BL/6 mice, while a susceptible phenotype is observed in BALB/c mice [175-179]. While it is known that IL-4 generally induces a Th2 response, some reports have recently demonstrated that an early production of IL-4 at the site of infection, may in fact drive a Th1 response, under the instruction of dendritic cells [135, 235]. This is further supported by the fact that both BALB/c and C57BL/6 mouse strains secrete IL-4 early after *L. major* infection [78]. Furthermore, a recent study demonstrated that in the presence of *L. major* IL-81 promastigotes, keratinocytes are rapidly induced to secrete immunomodulatory mediators including IL-12, IL-1 β , IL-4, and IL-6, and suggested that the keratinocytes may be providing the early IL-4 that instructs DCs to drive the Th1 response [239].

The IL-4R α chain controls the biological functions of IL-4 and IL-13 [98], thus in order to block IL-4/IL-13 signalling on keratinocytes, we can block the signalling receptor on this cell. To ascertain whether IL-4/IL-13 signalling by keratinocytes is crucial in the outcome of cutaneous *Leishmania* disease, we generated a keratinocyte-specific IL-4R α deficient mouse on a BALB/c and C57BL/6 genetic background (KRT14^{cre}IL-4R α ^{-/lox} mice) and infected these mice with *L. major* IL-81 or LV39 to analyse disease progression and host immune responses. We found that in the footpad infection model, abrogated IL-4/IL-13 signalling via IL-4R α on

keratinocytes, KRT14^{cre}IL-4Rα^{-lox} BALB/c mice developed increased footpad swelling, high parasite burdens, both Th1 and Th2 immune responses, and high type 1 and type 2 antibody responses, similar to littermate control mice. On a C57BL/6 genetic background, in the footpad infection model, we saw that the deletion of IL-4/IL-13 signalling via IL-4Rα on keratinocytes resulted in KRT14^{cre}IL-4Rα^{-lox} C57BL/6 mice developing low footpad swelling, low parasite burdens, stronger Th1 immune responses, and low type 1 and type 2 antibodies, similar to littermate control mice on the C57BL/6 genetic background. In the ear infection model, the absence of signalling of IL-4/IL-13 via IL-4Rα on keratinocytes resulted in KRT14^{cre}IL-4Rα^{-lox} BALB/c mice showing increased ear swelling, high parasite burdens, high Th1 and Th2 immune responses, and high type 1 and type 2 antibody responses, similar to littermate control mice. Additionally, the absence of IL-4Rα-responsive keratinocytes contributed to the control of parasites in the ears of these KRT14^{cre}IL-4Rα^{-lox} BALB/c mice. Finally, on a C57BL/6 genetic background, in the ear infection model, we saw that abrogated IL-4/IL-13 signalling via IL-4Rα on keratinocytes resulted in KRT14^{cre}IL-4Rα^{-lox} C57BL/6 mice developing low ear swelling, low parasite burdens, stronger Th1 immune responses, and low type 1 and type 2 antibodies, similar to littermate control mice on the C57BL/6 genetic background. While immune responses may have been influenced in some cases, KRT14^{cre}IL-4Rα^{-lox} mice on the BALB/c genetic background were still non-healing, and KRT14^{cre}IL-4Rα^{-lox} mice on the on the C57BL/6 genetic background could still heal lesions.

2.2 Materials

All general reagents were purchased from either of the following suppliers: Merck Chemicals (Pty) Ltd.; Gibco Life Technologies; Corning, Invitrogen Life Technologies, Roche, and Sigma Aldrich. Molecular biology reagents were purchased from ThermoScientific, BD Bioeciences and Fermentas Life Sciences. See Appendix for reagents for all experiments.

2.3 Methods

2.3.1 Ethical statement

All mouse experiments and protocols were performed in strict accordance with the South African national guidelines, as well as the Animal Research Ethics Committee of the Faculty of Health Sciences, University of Cape Town (license no. 015/034). All efforts were made to minimise and reduce suffering of animals.

2.3.2 Generation and genotyping of KRT14^{cre}IL-4Rα^{-lox} mice

Keratinocyte cell-specific IL-4Rα-deficient (KRT14^{cre}IL-4Rα^{-lox}) BALB/c mice were generated using the *Cre/loxP* system, and characterized by our laboratory (unpublished). Briefly, KRT14^{cre} mice (Stock Tq (KRT14cre) 1Amc/J-Jackson laboratory) were crossed with IL-4Rα^{-/-} BALB/c mice [233] and transgenic IL-4Rα^{lox/lox} mice [134] to generate hemizygous KRT14^{cre}IL-4Rα^{-lox} BALB/c mice. These KRT14^{cre}IL-4Rα^{-lox} mice were also generated on a C57BL/6 background in our laboratory. All cutaneous leishmaniasis experiments included the following mouse groups (BALB/c or C57BL/6 background): KRT14^{cre}IL-4Rα^{-lox}, hemizygous (also wild-type) littermate controls (IL-4Rα^{-lox}), wild-type BALB/c, global IL-4Rα^{-/-} and C57BL/6 mice. All mice were kept under specific pathogen-free conditions, in individually ventilated cages. Experimental mice were age and sex-matched, and used between 8 to 10 weeks of age. Genotyping of KRT14^{cre}IL-4Rα^{-lox} BALB/c and C57BL/6 mice was carried out by Miss Munadia Ansarie at the Division of Immunology, using specific primers: KRT14 P1, 5'- TTC CTC AGG AGT GTC TTC GC; KRT14 P2, 5'- GTC CAT GTC CTT CCT GAA GC; KRT14 P3, 5'- CAA ATG TTG CTT GTC TGG TG; KRT14 P4, 5'- GTC AGT CGA GTG CAC AGT TT. IL-4Rα deletion was confirmed with flow cytometry, staining for the IL-4Rα on ear-isolated keratinocytes (Joint experiment by the author and collaborators at the University Of Lausanne, Switzerland) (unpublished). The isolation of keratinocytes from the ear, and staining for IL-4Rα were performed by the author, assisted by Dr. M. Descatoire, at the University Of Lausanne, Switzerland, in the Tacchini-Cottier laboratory. The protocols were developed in the Tacchini-Cottier laboratory. Briefly, ears were digested in complete Dulbecco's Modified Eagle's Medium (cDMEM) containing liberase (TL research grade, Roche). The reaction was stopped with EDTA, ear samples were crushed through a 40 μM filter, and the supernatant centrifuged. Cells were then counted and labelled. Labelling was performed by adding the antibodies for 20 minutes on ice, followed by the addition of the secondary antibody. Keratinocytes were gated as CD45⁻ and were double positive for CD49 and K14. Antibodies included CD45-PerCP, CD49f-FITC, mouse anti-Keratin 14 and Goat anti-mouse A555. CD45⁻ CD49⁺ K14⁺ keratinocytes were then stained for the presence of IL-4Rα with IL-4Rα-PE, and samples were acquired on the Fortessa machine (BD, San Jose, CA, USA). Flow data was analyzed using Flowjo software (www.flowjo.com/) (Treestar, Ashland, OR, USA).

2.3.3 *L. major* infection

L. major strains: LV39 sub strain obtained from American Type Culture Collection (ATTC) 50132 strain (MRHO/SU/59/P) and LV39 sub strain (MRHO/SV/59/P) obtained from the University of Lausanne [180, 280]; *L. major* IL-81 (MHOM/IL/81/FEBNI); and GFP-expressing *L. major* IL-81 (MHOM/IL/81/FEBNI) (kind gift from Prof. Heidrun Moll, University of Würzburg, Germany) were maintained via continuous passage through BALB/c mice [233], and *in vivo* cultures were incubated in Schneiders media (Sigma-Aldrich) supplemented with 20 % fetal calf serum (FCS) in a tissue flask (Corning). Parasites were prepared for infection as previously described [233]. Briefly, parasites were harvested from the footpad lesion and popliteal lymph node of an infected mouse, followed by *in vitro* culturing at 37°C, in a tissue culture flask containing 10 ml Schneiders' medium supplemented with 20 % FCS (SCH+FCS medium). Parasite growth was monitored over a 7 day period, after which confluent parasites were fixed in 2% glutaraldehyde, counted using a Neubauer cytometer slide, and prepared for murine infection studies. Mice were anaesthetised prior to subcutaneous inoculation with 2×10^5 (IL-81) or 2×10^6 (LV39) stationary phase metacyclic promastigotes, into the left hind footpad, contained in a volume of 50 µl of phosphate buffered saline (PBS) [132, 135, 233]. Disease progression was monitored weekly, by measuring change in swelling of infected footpads, using a Mitutoyo micrometer caliper (Brütsch, Zürich, Switzerland). Alternatively, mice received an intradermal inoculation with 1×10^4 stationary phase metacyclic promastigotes, into the left ear, contained in a volume of 10 µl of PBS [281-283]. Disease progression was monitored weekly, by measuring change in diameter of lesion of infected ear, using a vernier calliper (South Africa). In the footpad infection model, mice infected with *L. major* LV39 were sacrificed at 8 weeks post infection (p.i.) whilst mice infected with *L. major* IL81 strains were sacrificed at 6 weeks p.i. In the ear infection model, mice were sacrificed at 8 weeks p.i. regardless of infecting strain.

2.3.4 Preparation of soluble *Leishmania major* antigen (SLA)

Parasites were recovered from *in vitro* cultures as described in Section 2.3.3. SLA was prepared from these stationary phase metacyclic promastigotes as follows: parasites were centrifugated at 5000 rpm for 10 min at 4°C, the pellet re-suspend in 1 ml PBS-containing cocktail protease inhibitor (Sigma-Aldrich) and transferred to a Greiner tube, the re-suspension sonicated to lyse the cells (4 x 20 sec with 20 sec rest period), the solution transferred to a sterile eppendorf and centrifuge at 8000 rpm for 10 min at 4 °C, and the supernatant filtered through a 0.22 µM filter. The concentration of the SLA was determined using a Pierce® bicinchoninic acid protein assay

(BCA), which was carried out as recommended by the manufacturer. A 2 mg/ml bovine serum albumin (BSA) stock solution provided was used to produce protein standards. SLA was stored in aliquots at -80 °C until required.

2.3.5 Determination of viable parasite burden

The limiting dilution assay (LDA) was used to quantify viable parasite burden [233]. The infected footpad, ear, and spleen were collected in 2 ml SCH+FCS medium, whilst draining lymph nodes were collected in 1 ml cDMEM. Draining lymph nodes were processed through a 40 µm strainer to obtain single cell suspensions in a final volume of 6.4 ml (100 µl equivalent to 2^6 parasites). Two-fold serial dilutions were prepared in 96 well NUNC flat-bottom plates using SCH+FCS medium in a final volume of 100 µl. 7 days post-incubation at 37°C, individual wells were examined and scored microscopically for the presence or absence of parasite growth.

2.3.6 Isolation and stimulation of draining lymph node cells

Single cell suspensions (prepared as described in Section 2.3.5) were centrifuged at 1500 rpm for 5 min at 4°C and filtered through 40 µm filter. Pelleted cells were re-suspended in 3 ml cDMEM, and viable cells were counted using Trypan Blue to exclude dead cells. Cells were made to 1×10^7 (100 µl equivalent to 1 million cells). The cells were then cultured in triplicate at 1×10^6 /ml in 48-well flat-bottom plates, and left unstimulated, or stimulated with 20 µg/ml anti (α)-CD3 or 50 µg/ml soluble *L. major* antigen (SLA). Cells were incubated at 37°C and 5% CO₂. After 48 hrs, plates were centrifuged and supernatants were removed and used for the detection of the following cytokines: IFN-γ; IL-4; and IL-13, using enzyme-linked immunosorbent assay (ELISA).

2.3.7 Flow cytometry

Draining lymph node cells from *L. major* infected mice were prepared (as described in Sections 2.3.5 and 2.3.6) and seeded (in a 96 well NUNC plate) for intracellular cytokine (IC) staining (2×10^6 cells per well), and cell populations (1×10^6 cells per well). For cell populations, cells were centrifuged in NUNC V-bottom plates, and the pellets were re-suspended and stained in 50 µl antibody mix for 30 min in foil at 4°C. After incubation, 150 µl FACS buffer was added, and cells were spun down, followed by the re-suspension of cells in 2% paraformaldehyde in PBS for fixing, incubated in foil for 30 min at 4°C. Cells were thereafter washed in PBS, followed by final re-suspension in 200 µl FACS buffer. The expression of surface markers on cell populations (T cells, B cells, dendritic cells and macrophages) were

identified using monoclonal antibodies labelled with one of the following: fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC) or peridinin chlorophyll protein (PerCP). T and B cell panel included: CD3-FITC; CD4-PE; CD8-APC; CD19-PerCpCy5.5. Dendritic cell and macrophage panel included: CD11c-APC; CD11b-PE; MHCII-FITC. Each mix also included 1% rat serum and 10 µg FC gamma receptor blocker (*Fcγ*). For IC staining, cells were seeded in 96 well U-bottom plates and stimulated at 37°C for 2 hours in cDMEM with 50ng/ml phorbol myristate acetate (PMA) and 250 ng/ml ionomycin (IONO), followed by the addition of 200 µM monensin, for 4 hrs. Following surface staining in 50 µl antibody mix (CD3-FITC, CD4-PerCP, CD8-APC, 1% rat serum, 10 µg *Fcγ*) for 30 min in foil at 4°C, cells were washed and fixed as described above, and next permeabilized with permeabilization buffer for 30 min in foil at 4°C. Intracellular cytokines were added (all cytokine antibodies IFN- γ , IL-4 and IL-13 on PE channel), for 60 min in foil at 4°C, followed by a wash and final re-suspension in FACS buffer, ready for acquisition. Acquisition of cells was completed on FACS Calibur machine (BD Immunocytometry systems, San Jose, CA, USA). Flow data was analyzed using Flowjo software (www.flowjo.com/) (Treestar, Ashland, OR, USA). Representative flow cytometry plots and gating strategies are available in the Supplementary Figures section.

2.3.8 Detection of antibody titres with ELISA

Antibody ELISAs were carried out as previously described [233]. Briefly, at the completion of each experiment, blood samples were retrieved via cardiac puncture and collected into serum separator tubes (BD Bioscience, San Diego, CA). The samples were centrifuged at 8000 rpm for 10 min 4°C. The indirect ELISA was used to detect antigen-specific levels of IgG2a and IgG1. Nunc MaxiSorp flat-bottom 96 well ELISA plates were used. Wells were coated overnight at 4°C with SLA diluted in carbonate coating buffer (final concentration: 5 µg/ml). Following overnight incubation, the wells were blocked with 2% milk powder for 3 hrs at 37°C. Plates were washed 3 times and the serum (diluted 1 in 10 with dilution buffer) was added in 3-fold serial dilutions, followed by overnight incubation at 4°C. Plates were washed as above, and alkaline phosphatase (AP)-labelled specific antibodies were added for 1 hr at 37°C. The sandwich ELISA was used to measure total IgE. Wells were coated with IgE clone 84.1C in carbonate coating buffer and incubated overnight at 4°C. Wells were washed, blocked, and sample added, as described above, followed by the addition of Rat- α -mouse IgE AP. After incubation with AP-labelled antibody, plates were washed and 4-Nitrophenylphosphate (PNP) added (1 mg/ml dissolved in substrate buffer). Optical density was measured at 405 nm with

492 nm as a reference wavelength. Micro-titre plate readings were carried out with a VersaMax™ ELISA plate reader.

2.3.9 Detection of cytokine production with ELISA

The sandwich ELISA [233] was used to determine the concentration of the following cytokines: IFN- γ ; IL-4; and IL-13 in cell supernatants obtained in Section 2.3.6. ELISA plates were coated with purified anti-IFN- γ (AN18KL6), anti-IL-4 (11B11), or anti-IL-13 (R&D MAB413), diluted in PBS coating buffer, and incubated overnight at 4°C. Wells were then blocked and washed as described above in Section 2.2.9, flowed by the addition of purified recombinant cytokine standards IFN- γ , IL-4, IL-13, or cell supernatants (Section 2.3.6) added in 3-fold serial dilutions and incubated overnight at 4°C. Wells were washed, and biotinylated anti-mouse secondary antibodies specific for each cytokine, was added for 3hrs at 37°C. Wells were then washed and incubated with AP-labelled streptavidin for 1 hr at 37°C, followed by incubation with PNP Substrate as above in Section 2.3.8. The reaction was stopped with 1 M NaOH and absorbance was read as described in Section 2.3.8. The detection limits for the ELISAs were approximately 0.13 ng. Micro-titre plate readings were carried out with a VersaMax™ ELISA plate reader.

2.3.10 Statistics

Statistical analysis was carried out using GraphPad Prism 5 software (<http://www.prism-software.com>). The data was calculated as mean \pm SEM. Statistical significance was determined using the unpaired Student's *t* test or 2-way ANOVA with Bonferroni's post-test (unless otherwise stated), defining differences to IL-4R $\alpha^{-/lox}$ as significant (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$).

2.4 Results

2.4.1 Genotypic and phenotypic characterization of KRT14^{cre}IL-4R $\alpha^{-/lox}$ mice

Genetically modified BALB/c mice expressing Cre-recombinase under control of the keratinocyte cell-specific promoter KRT14 (Jackson laboratory) were inter-crossed with IL-4R $\alpha^{-/-}$ BALB/c mice [233] and IL-4R $\alpha^{lox/lox}$ BALB/c [134] to generate KRT14^{cre}IL-4R $\alpha^{-/lox}$ mice (Supplementary Figure 1A). KRT14^{cre}IL-4R $\alpha^{-/lox}$ BALB/c mice were identified by PCR genotyping (Supplementary Figure 1B). Analysis of IL-4R α surface expression on isolated ear keratinocytes by flow cytometry demonstrated that IL-4R α was efficiently depleted in KRT14^{cre}IL-4R $\alpha^{-/lox}$ BALB/c mice as seen by the shift of IL-4R α expression away from wild-type BALB/c mice (Supplementary Figure 1C). The deletion of IL-4R α on KRT14^{cre}IL-4R $\alpha^{-/lox}$

^{/lox} C57BL/6 mice was also confirmed by PCR (data not shown) and flow cytometry ((Supplementary Figure 1D).

2.4.2 KRT14^{cre}IL-4R α ^{-lox} BALB/c mice remain susceptible to *L. major* infection in the footpad model similar to littermate control BALB/c mice

In order to determine whether the signalling molecule IL-4R α present on keratinocytes at the site of infection is important in disease outcome, KRT14^{cre}IL-4R α ^{-lox} BALB/c mice were infected subcutaneously in the left hind footpad with stationary phase metacyclic promastigotes, either 2×10^5 *L. major* of the highly virulent IL-81 strain (Figure 2.1A). The footpad infection murine model for leishmaniasis has been well established in our laboratory [94, 132, 135, 284], and in other groups [285-287]. As expected, and demonstrated in previous reports [132, 233], IL-4R α ^{-/-} BALB/c and C57BL/6 mice controlled the development of lesions during acute infection with *L. major* IL-81 (Figure 2.1A), correlating with the low parasite burdens observed in the homogenised infected footpads, draining popliteal lymph nodes, and spleens, as determined by limiting dilution assay (LDA). Mice were also infected with 2×10^6 *L. major* LV39 ATTC strain (Figure 2.1B). Interestingly, during infection with this strain, the IL-4R α ^{-/-} BALB/c, still exhibiting swelling significantly lower to the littermate (IL-4R α ^{-lox}) control mice, seemed to have a swelling almost identical to that of the wild-type BALB/c mice (Figure 2.1B). Parasite burdens for IL-4R α ^{-/-} BALB/c mice infected with *L. major* LV39 ATTC were unexpectedly significantly higher in the draining lymph node, in comparison to the littermate control mice (Figure 2.1B). The discrepancy of the phenotype of the IL-4R α ^{-/-} BALB/c mice, is due to the difference in sub strain and source of LV39 [288]. In both sets of infections, wild-type BALB/c mice developed progressive footpad swelling, as expected of this genetically susceptible strain, which was significantly lower than the littermate control IL-4R α ^{-lox} mice (Figure 2.1A and 2.1B). We included genetically susceptible wild-type BALB/c and littermate control mice in all our experiments, due to a gene-dosage effect for IL-4R α expression that can cause differences in their immunological response [289]. Holmdahl *et al.* also explains the need for littermate control mice to be included in experiments [290]. Importantly, during both *L. major* IL-81 and *L. major* LV39 infections, the KRT14^{cre}IL-4R α ^{-lox} BALB/c mice developed progressive footpad swelling similar to littermate control mice, and exhibited similarly high parasite burdens in the infected footpads, draining popliteal lymph nodes, and spleens (Figure 2.1). These results suggested that deletion of the IL-4R α on

keratinocytes in BALB/c mice does not affect the progression of *L. major* disease in the classical footpad model.

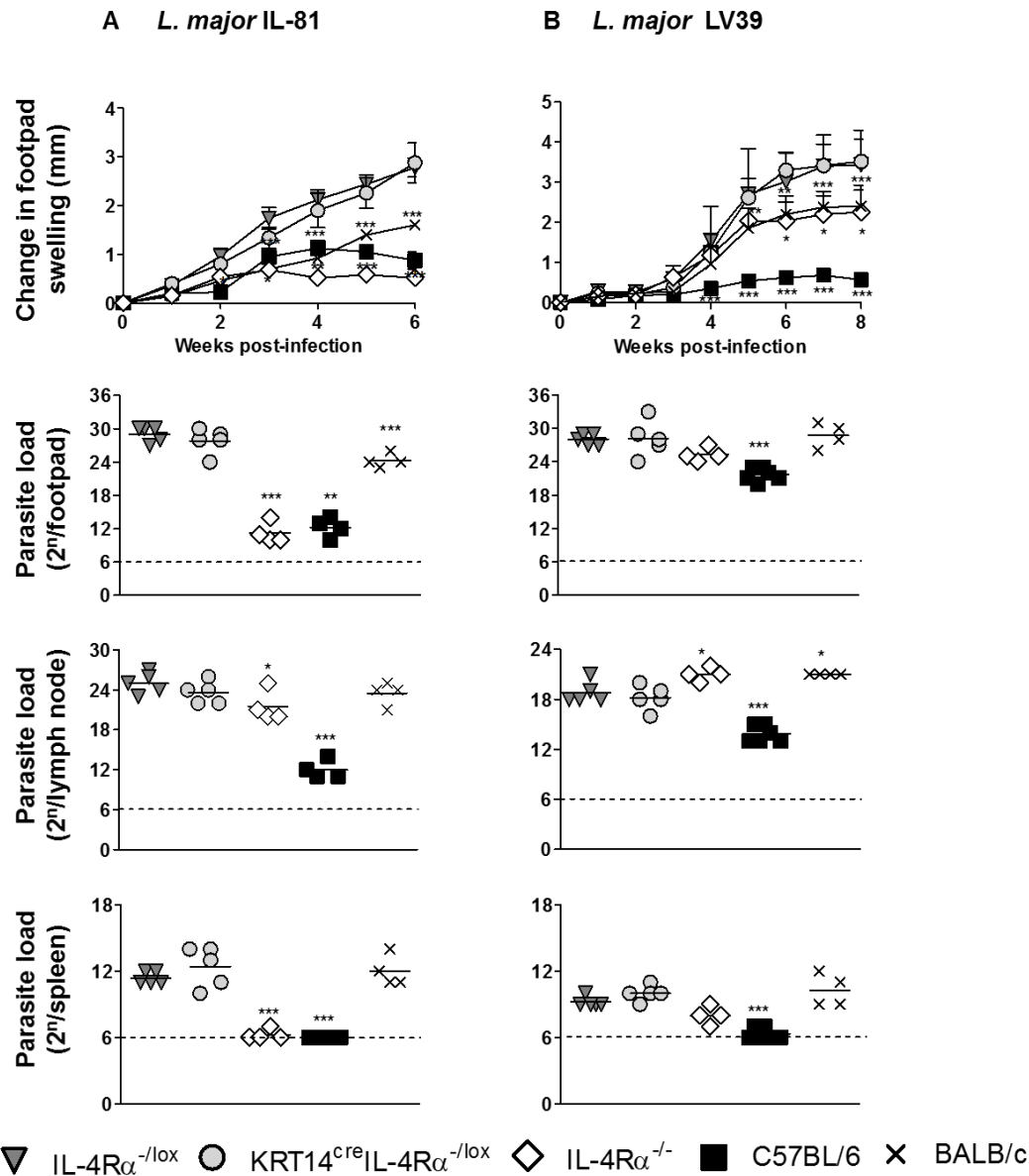


Figure 2.1 KRT14^{cre}IL-4R $\alpha^{-/-lox}$ BALB/c mice are clinically susceptible to *L. major* similar to littermate control mice in the footpad murine model. Mice were infected subcutaneously in the left hind footpad with stationary phase metacyclic *L. major* promastigotes at either (A) 2×10^5 IL-81 or (B) 2×10^6 LV39. The change in footpad swelling in mm was measured at weekly intervals (5-7 mice per group). Parasite burden was determined at week 6 for (A) and week 8 for (B), by limiting dilution of homogenized footpads, single-cell suspensions of the draining popliteal lymph nodes, and homogenised spleens. A representative of two individual experiments is shown with mean values \pm SEM (Statistical analysis was performed with comparisons to the control IL-4R $\alpha^{-/-lox}$ littermate mice group, as significant (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). Experiments and analysis were carried out by Kaya Gqada and Dr R. Hurdal, and the figure was compiled by the author.

2.4.3 KRT14^{cre}IL-4R α ^{-lox} BALB/c mice exhibit a cell population repertoire, cytokine profile, and antibody responses model similar to littermate control BALB/c mice during *L. major* infection in the footpad

Wild-type BALB/c mice are susceptible to *L. major* infection and demonstrate a Th2 polarised immune response [176, 291]. We evaluated the immune response of KRT14^{cre}IL-4R α ^{-lox} BALB/c mice during *L. major* infection to determine whether these mice elicited a polarised Th2 immune response, since they presented as clinically susceptible mice. Cell populations infiltrating the draining lymph node of KRT14^{cre}IL-4R α ^{-lox} BALB/c mice as determined using surface staining, showed no differences when compared to littermate control mice (Figure 2.2A and 2.2B). Percentages of the CD3⁺CD4⁺ T helper cell were similar in all mouse groups except C57BL/6 mice, which showed significantly lower levels. The percentage of CD3⁺CD8⁺T cytotoxic cells present in the draining lymph node were similar in all groups of mice. CD19⁺ B lymphocytes were similarly high in all mouse groups, however, only C57BL/6 mice had a significantly higher percentage of these cells compared to littermate controls. KRT14^{cre}IL-4R α ^{-lox} BALB/c mice showed similar levels of dendritic cells and macrophages when compared to littermate control mice, similar to IL-4R α ^{-/-} and wild-type BALB/c mice, with C57BL/6 mice having a higher percentage that was not significant (Figure 2.2B). Levels of macrophages were similar in all groups of mice, except the C57BL/6 mice, which had significantly more macrophages than the littermate control mice (Figure 2.2B). Cell numbers of the different populations followed the same pattern as with percentages (Data not shown). Collectively, these results suggest that cellular infiltration in the lymph node occurred independently of IL-4R α -responsive keratinocytes during *L. major* infection in KRT14^{cre}IL-4R α ^{-lox} BALB/c mice.

To investigate the impact of IL-4R α deficiency on keratinocytes on the cytokine production by CD4⁺ T helper cells during infection, single LN cell suspensions (as described above) were stimulated with PMA/Ionomycin/Monensin and stained for intracellular cytokine production (Figure 2.2C). Resistant C57BL/6 mice illustrated significantly higher levels of IFN- γ , and significantly lower levels of IL-4 and IL-13, as expected [135], in comparison to littermate control mice. KRT14^{cre}IL-4R α ^{-lox} BALB/c and remaining mouse groups, IL-4R α ^{-/-} and wild-type BALB/c mice, showed similar levels of all three cytokines., with IL-4R α ^{-/-} mice having significantly lower levels of IL-13, as compared to littermate control mice. LN cells were also stimulated with soluble *leishmanial* antigen (SLA) or α -CD3 (strong mitogen for T cells) to demonstrate cytokine production (Figure 2.2D-F). Similar levels of IFN- γ (Figure 2.2D), IL-4 (Figure 2.2E) and IL-13 (Figure 2.2F) were observed in KRT14^{cre}IL-4R α ^{-lox} BALB/c and

littermate control mice. In contrast to CD4⁺ T cell cytokine production (Figure 2.2C), C57BL/6 mice showed significantly lower levels of all three cytokines, when LN cells were stimulated with α -CD3 as compared to littermate control mice (Figure 2.2D-F). Stimulation of LN cells from wild-type BALB/c mice with both α -CD3 and SLA illustrated similar levels of all cytokines in comparison to littermate control mice. When stimulated with α -CD3, IL-4R α ^{-/-} mice produced similar levels of IFN- γ (Figure 2.2D), and significantly lower levels of IL-4 (Figure 2.2E) and IL-13 in comparison to littermate control mice (Figure 2.2F). SLA stimulation of LN cells from IL-4R α ^{-/-} mice produced lower levels of IFN- γ which was not significant (Figure 2.2D), significantly lower levels of IL-4 (Figure 2.2E) and lower but not significant levels of IL-13 (Figure 2.2F) as compared to littermate control mice. The data suggests that IL-4R α responsive keratinocytes do not play a role in driving a Th1 polarised response during *L. major* infection in the footpad mouse model.

The quantification of cytokine production after *ex vivo* stimulation may not provide a true indication of the Th1 and Th2 responses *in vivo* [291]. Furthermore, it is known that IL-4 promotes isotype switching to IgG1 and IgE, and IgG2a levels correlates to the activity of IFN- γ *in vivo* [292]. For these reasons, we measured SLA-specific Type 1 (IgG2a) and type 2 (IgG1 and Total IgE) antibody titres in the mouse sera by ELISA, 6 weeks post-infection with *L. major* IL-81. KRT14^{cre}IL-4R α ^{-/lox} BALB/c and littermate control mice illustrated similar higher levels of IgG2a (Figure 2.2G), similar lower levels IgG1 (Figure 2H) and similar higher levels of total IgE (Figure 2.2I). Compared to littermate control mice, IL-4R α ^{-/-} had significantly lower levels of IgG2a, as did wild-type BALB/c mice, as well as significantly lower levels of IgG1 and total IgE, similar to C57BL/6 mice. Significantly more IgG1 and IgE was produced by wild-type BALB/c mice in comparison to the littermate control mice. Similar observations were seen with mice during an *L. major* LV39 ATTC infection (data not shown). Collectively, the data suggested that systemic antibody responses in KRT14^{cre}IL-4R α ^{-/lox} BALB/c mice were unaffected by the deletion of the IL-4R α signalling receptor on keratinocytes, during infection with *L. major* IL-81 and LV39 ATTC, in the footpad infection model.

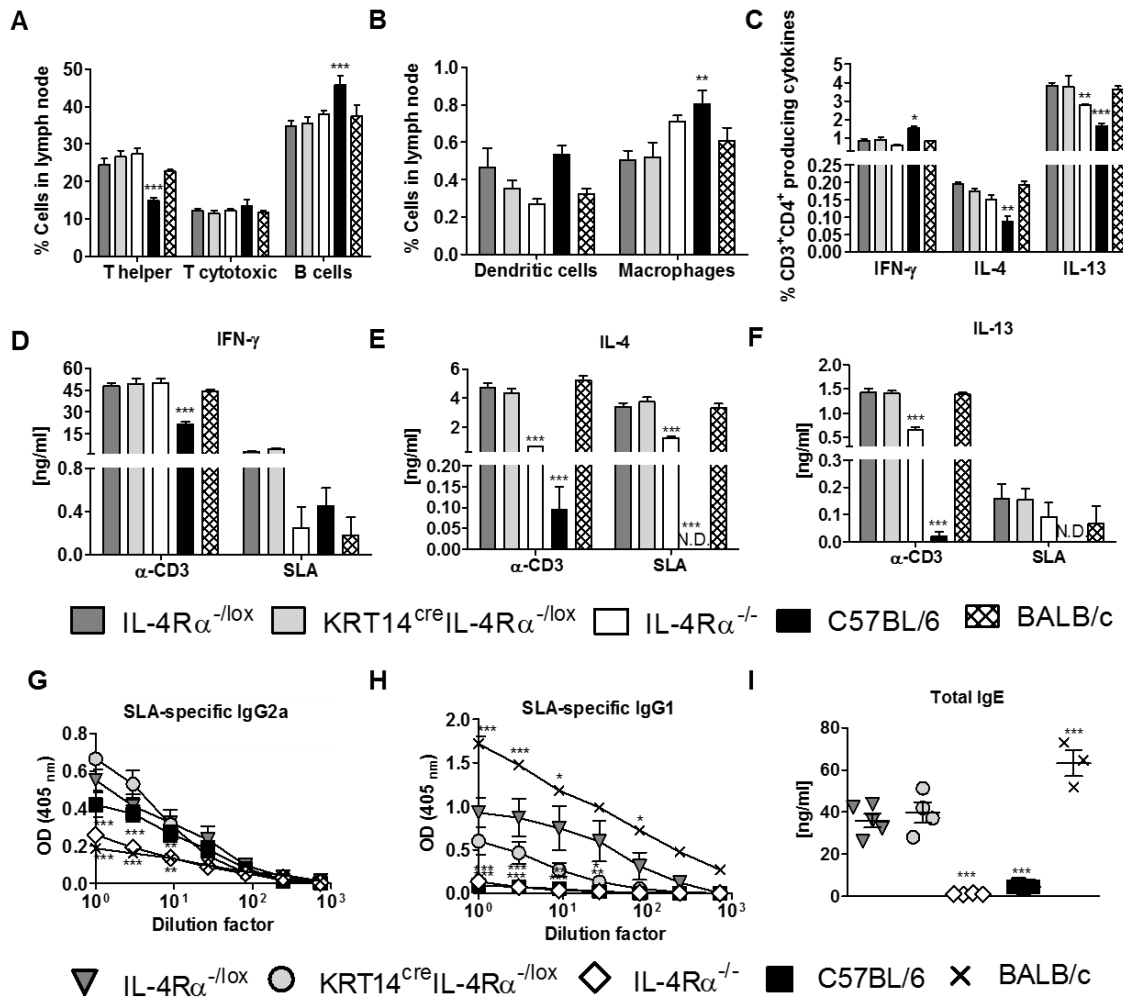


Figure 2.2 KRT14^{cre}IL-4Rα^{-/-} BALB/c mice develop an immune response similar to littermate control mice during *L. major* IL-81 infection in the footpad murine model. At 6 weeks post-infection, mice were sacrificed and popliteal draining lymph nodes retrieved. Single cell suspensions obtained from the lymph nodes were extracellularly stained for the following populations: (A) T helper (CD3⁺CD4⁺), T cytotoxic (CD3⁺CD8⁺), B cells (CD19⁺B220⁺); (B) dendritic cells (CD11c^{hi}MHCII^{hi}) and macrophages (CD11b^{hi}CD11c^{hi}MHCII^{hi}), and also (C) stimulated with PMA/ionomycin/monensin, followed by staining for intracellular cytokine production. Cells were re-stimulated with α-CD3 and soluble leishmanial antigen (SLA) for 72 hours, after which cytokine production was measured with ELISA for (D) IFN-γ, (E) IL-4 and (F) IL-13. (G) *L. major*-specific type 1 (IgG2a) and (H-I) type 2 antibodies (IgG1 and total IgE) were measured in sera from 6 week infected mice by ELISA. Data is representative of two experiments. Statistical analysis was performed with comparisons to the control IL-4Rα^{-/-} littermate mice group, as significant (*, p<0.05, **, p<0.01; ***, p<0.001). Experiments and analysis were carried out by Kaya Gqada and Dr R. Hurdal, while figure was compiled by the author.

2.4.4 Resistant phenotype is maintained in KRT14^{cre}IL-4Rα^{-/-} C57BL/6 mice during *L. major* infection in the footpad model similar to littermate control C57BL/6 mice

C57BL/6 mice are genetically resistant to *L. major*, mounting a Th1 polarised response during infection [176]. A recent study has, however, demonstrated that early neutralization of IL-4 in these C57BL/6 mice resulted in a switch to Th2 immune response [239]. Furthermore, the authors suggested that the IL-4 driving characteristic Th1 response in C57BL/6 mice may be

secreted by keratinocytes at the site of infection[239]. We therefore decided to investigate whether the absence of the signalling molecule IL-4R α (through which IL-4 and IL-13 signals) on keratinocytes in C57BL/6 mice, would result in these resistant mice displaying susceptibility during *L. major* infection. KRT14^{cre}IL-4R α ^{-lox} on a C57BL/6 background were infected subcutaneously in the left hind footpad as described above with *L. major* IL-81 (Figure 2.3A) and *L. major* LV39 (MRHO/SV/59/P strain was used for all experiments from this point onwards, replacing the ATTC (MRHO/SU/59/P) strain) (Figure 2.3B). For these infections, the littermate (IL-4R α ^{-lox}) control mice and IL-4R α ^{-/-} were both on C57BL/6 genetic backgrounds. Importantly, this is the first report on the use of IL-4R α ^{-/-} on a C57BL/6 genetic background in *L. major* murine models. Wild-type BALB/c mice were included as a susceptible (Th2-driven) control. Swelling progression in KRT14^{cre}IL-4R α ^{-lox} C57BL/6, littermate control mice and IL-4R α ^{-/-} mice were similar during *L. major* IL-81 infection (Figure 2.3A). During infection with *L. major* IL-81, IL-4R α ^{-/-} mice had completely healed by week 6, and all mouse groups except wild-type BALB/c mice had completely healed by week 8. Similarly, the swelling progression of KRT14^{cre}IL-4R α ^{-lox} C57BL/6, littermate control mice and IL-4R α ^{-/-} mice were comparable during *L. major* LV39 infection (Figure 2.3B). Wild-type BALB/c mice had significantly higher swelling compared to littermate control mice (which was on a C57BL/6 genetic background) during both *L. major* IL-81 and *L. major* LV39 infections. This is expected of the susceptible (wild-type BALB/c) versus resistant background (C57BL/6).

Parasite burdens in the infected footpad, during both *L. major* IL-81 and *L. major* LV39 infections, were similar in KRT14^{cre}IL-4R α ^{-lox} C57BL/6, littermate control mice and IL-4R α ^{-/-} mice, with a significantly higher burden seen in the wild-type BALB/c mice (Figure 2.3A and B). Lymph node parasite burden was similarly low in KRT14^{cre}IL-4R α ^{-lox} C57BL/6, littermate control mice and IL-4R α ^{-/-} mice, during both infections, however, during infection with *L. major* LV39, C57BL/6 mice appeared to have a slightly significantly lower burden. As expected, wild-type BALB/c mice demonstrated a significantly higher parasite burden during both infections as compared to littermate control mice (C57BL/6 genetic background) (Figure 2.3). During *L. major* IL-81 infection, C57BL/6 and IL-4R α ^{-/-} mice had no dissemination of the parasite to the spleen, while KRT14^{cre}IL-4R α ^{-lox} C57BL/6, littermate control and WT BALB/c mice showed dissemination (Figure 2.3A). This was unexpected as KRT14^{cre}IL-4R α ^{-lox} C57BL/6 and littermate control, having genetically resistant backgrounds, should have behaved like the C57BL/6 mice, and not presented in the spleen. During *L. major* LV39, only WT BALB/c mice displayed dissemination to the spleen, with an outlier in the IL-4R α ^{-/-} mouse

group having parasites in the spleen (Figure 2.3B). Collectively, the results indicate that deletion of the IL-4R α on keratinocytes in C57BL/6 mice does not alter the ability of animals to heal lesions, evident in control of footpad swelling and parasite burdens that are comparable to littermate control mice.

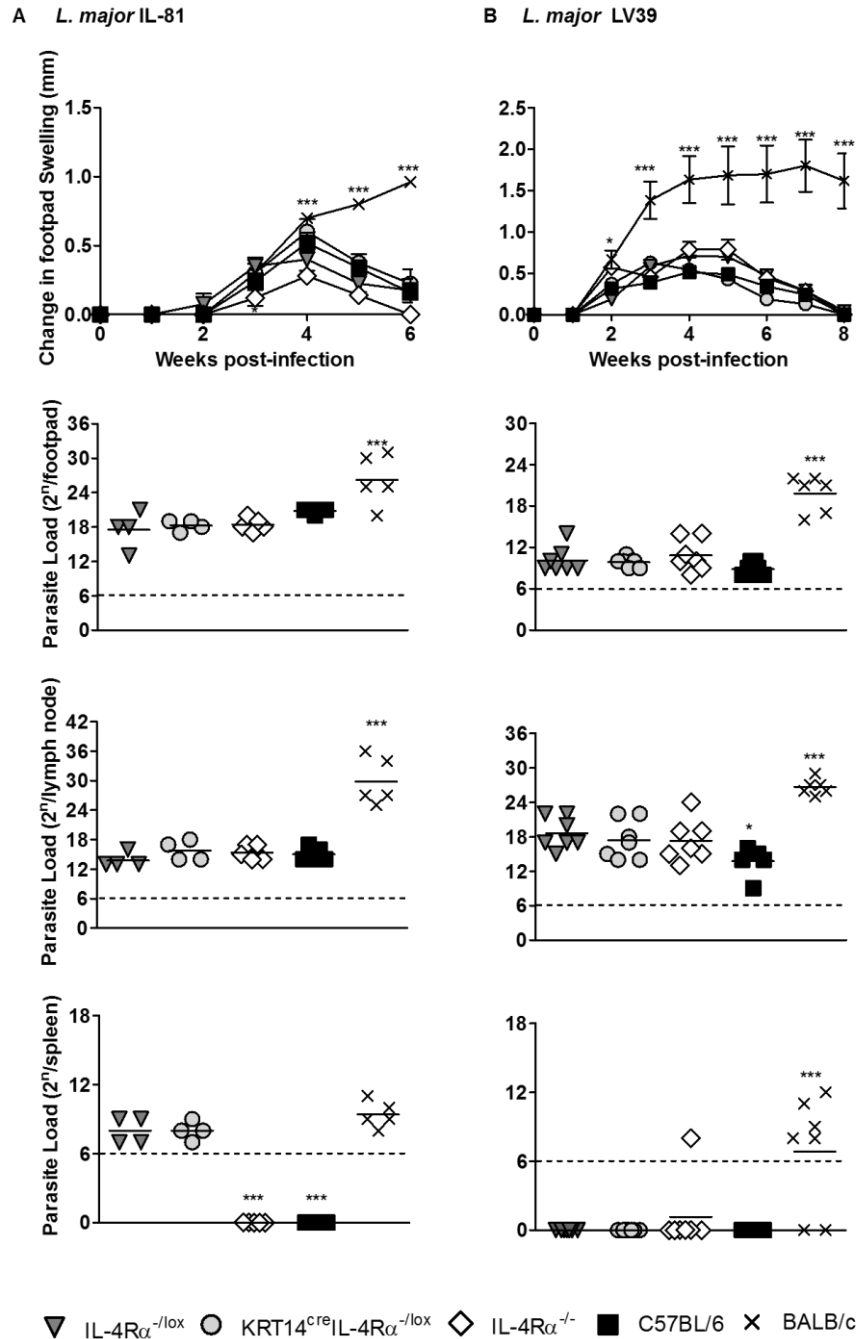


Figure 2.3 KRT14^{cre}IL-4R $\alpha^{-/lox}$ C57BL/6 mice are clinically resistant to *L. major* similar to littermate control mice in the footpad murine model. Mice were infected subcutaneously in the left hind footpad with stationary phase metacyclic *L. major* promastigotes at either (A) 2×10^5 IL-81 or (B) 2×10^6 LV39. The change in footpad swelling in mm was measured at weekly intervals (5-7 mice per group). Parasite burden was determined at week 6 for (A) and week 8 for (B), by limiting dilution of homogenized footpads, single-cell suspensions of the draining popliteal lymph nodes, and homogenised spleens. A representative of two individual experiments is shown with mean values \pm SEM (Statistical

analysis was performed with comparisons to the control IL-4R $\alpha^{-/lox}$ littermate mice group, as significant (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

2.4.5 Cellular and humoral response of KRT14^{cre}IL-4R $\alpha^{-/lox}$ C57BL/6 mice mimics Th1 immune response similar to resistant C57BL/6 mice during *L. major* infection in the footpad

To support the phenotypic data observed in KRT14^{cre}IL-4R $\alpha^{-/lox}$ C57BL/6 mice above in Figure 2.3, we evaluated the immune response of these mice during *L. major* IL-81 (Figure 2.4) and *L. major* LV39 infection (Figure 2.5) in the footpad. Extracellular staining of lymph node cells to show cell populations infiltrating the draining lymph node in KRT14^{cre}IL-4R $\alpha^{-/lox}$ C57BL/6 mice illustrated that percentages of CD3⁺CD4⁺ T helper and CD3⁺CD8⁺ T cytotoxic cells were similar to those in littermate control mice (Figure 2.4A, Figure 2.5A), as well as in IL-4R $\alpha^{-/-}$ mice and C57BL/6 mice. Wild-type BALB/c mice showed significantly higher percentages of CD3⁺CD4⁺ T helper and significantly lower CD3⁺CD8⁺ T cytotoxic cells compared to the littermate control mice. The percentages of the CD19⁺B220⁺ B cells in KRT14^{cre}IL-4R $\alpha^{-/lox}$ C57BL/6 were similar to littermate control mice, with a significantly higher percentage of B cells present in IL-4R $\alpha^{-/-}$ and C57BL/6 mice (Figure 2.4A, Figure 2.5A). Wild-type BALB/c mice showed lower B cell percentages during *L. major* IL-81 infection (Figure 2.4A), and significantly lower B cell levels during *L. major* LV39 infection (Figure 2.5A) as compared to littermate control mice. During *L. major* IL-81 infection, the percentage of dendritic cells and macrophages infiltrating the draining lymph node were similar in all mouse groups (Figure 2.4B), however, infection with *L. major* LV39 showed a downregulation of dendritic cells in C57BL/6 mice and upregulation of macrophages in wild-type BALB/c mice, with similar levels in KRT14^{cre}IL-4R $\alpha^{-/lox}$ C57BL/6, IL-4R $\alpha^{-/-}$ and littermate control mice (Figure 2.5B).

CD4⁺ T cell production of IFN- γ and IL-4 in KRT14^{cre}IL-4R $\alpha^{-/lox}$ C57BL/6 appeared to be similar to IL-4R $\alpha^{-/-}$ and littermate control mice, during both infections, with wild-type BALB/c mice showing upregulation in IL-4 during both infections, and an upregulation of IFN- γ during *L. major* LV39 infection (Figure 2.4C, Figure 2.5C). LN re-stimulations with α -CD3 demonstrated very high levels of IFN- γ in KRT14^{cre}IL-4R $\alpha^{-/lox}$ C57BL/6, IL-4R $\alpha^{-/-}$, C57BL/6 and littermate control mice during *L. major* IL-81 infection, but only wild-type BALB/c showed a significant difference, having lower levels of this cytokine (Figure 2.4D). Furthermore, extremely low levels of IL-4 and IL-13 levels were observed after α -CD3-LN stimulation in KRT14^{cre}IL-4R $\alpha^{-/lox}$ C57BL/6, IL-4R $\alpha^{-/-}$, C57BL/6 and littermate control mice

after *L. major* IL-81 infection, with wild-type BALB/c mice showing significant higher production of these two cytokines (Figure 2.4E-F), as previously demonstrated [291]. LN re-stimulation with SLA after *L. major* IL-81 infection demonstrated no difference in production of cytokines between mouse groups, except for C57BL/6 mice that appeared to produce significantly more IL-4 (Figure 2.4E). Re-stimulations of the LN after infection with *L. major* LV39 showed no differences in cytokine production in any of the C57BL/6 background groups, however, wild-type BALB/c mice was seen to have produced significantly more IL-4 and IL-13 after α -CD3-LN stimulation (Figure 2.5D-F). Together, these findings indicate that the IL-4R α -responsive keratinocytes are not required to drive a Th1 cellular response during *L. major* infection in the footpad model.

Infection with *L. major* IL-81 lead to low titres of SLA-specific Type 1 (IgG2a) and type 2 (IgG1 and total IgE) antibody titres in KRT14^{cre}IL-4R α ^{-/lox} C57BL/6, IL-4R α ^{-/-}, C57BL/6, comparable to littermate control mice (Figure 2.4G-I). A highly significant increase in IgG2a, IgG1, and total IgE was observed in wild-type BALB/c mice (Figure 2.4G-I) compared to littermate controls. We observed the same trends during *L. major* LV39 infection (Figure 2.5G-I). Our findings suggest that the humoral responses in KRT14^{cre}IL-4R α ^{-/lox} C57BL/6 mice are unaffected by the abrogation of IL-4R α on keratinocytes, during *L. major* infection in the footpad mouse model.

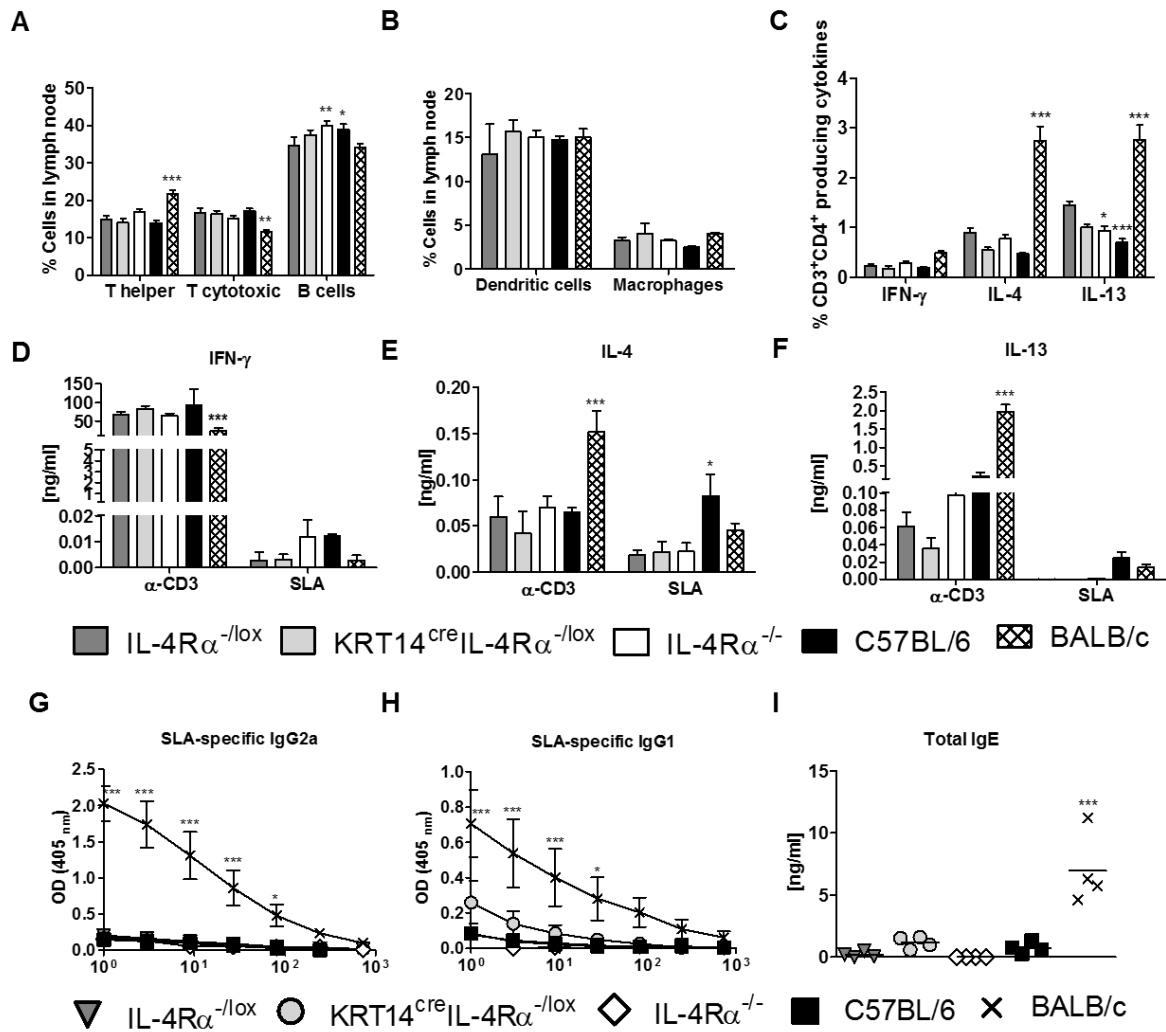


Figure 2.4 KRT14^{cre}IL-4Rα^{-/-} C57BL/6 mice develop an immune response similar to littermate control and resistant wild-type C57BL/6 mice during *L. major* IL-81 infection in the footpad murine model. At 6 weeks post-infection, mice were sacrificed and popliteal draining lymph nodes retrieved. Single cell suspensions obtained from the lymph nodes were extracellularly stained for the following populations: (A) T helper (CD3⁺CD4⁺), T cytotoxic (CD3⁺CD8⁺), B cells (CD19⁺B220⁺); (B) dendritic cells (CD11c^{hi}MHCII^{hi}) and macrophages (CD11b^{hi}CD11c^{hi}MHCII^{hi}), and also (C) stimulated with PMA/ionomycin/monensin, followed by staining for intracellular cytokine production. Cells were re-stimulated with α-CD3 and soluble leishmanial antigen (SLA) for 72 hours, after which cytokine production was measured with ELISA for (D) IFN-γ, (E) IL-4 and (F) IL-13. (G) *L. major*-specific type 1 (IgG2a) and (H-I) type 2 antibodies (IgG1 and total IgE) were measured in sera from 6 week infected mice by ELISA. Data is representative of two experiments. Statistical analysis was performed with comparisons to the control IL-4Rα^{-/-}lox littermate mice group, as significant (*, p<0.05, **, p<0.01; ***, p<0.001).

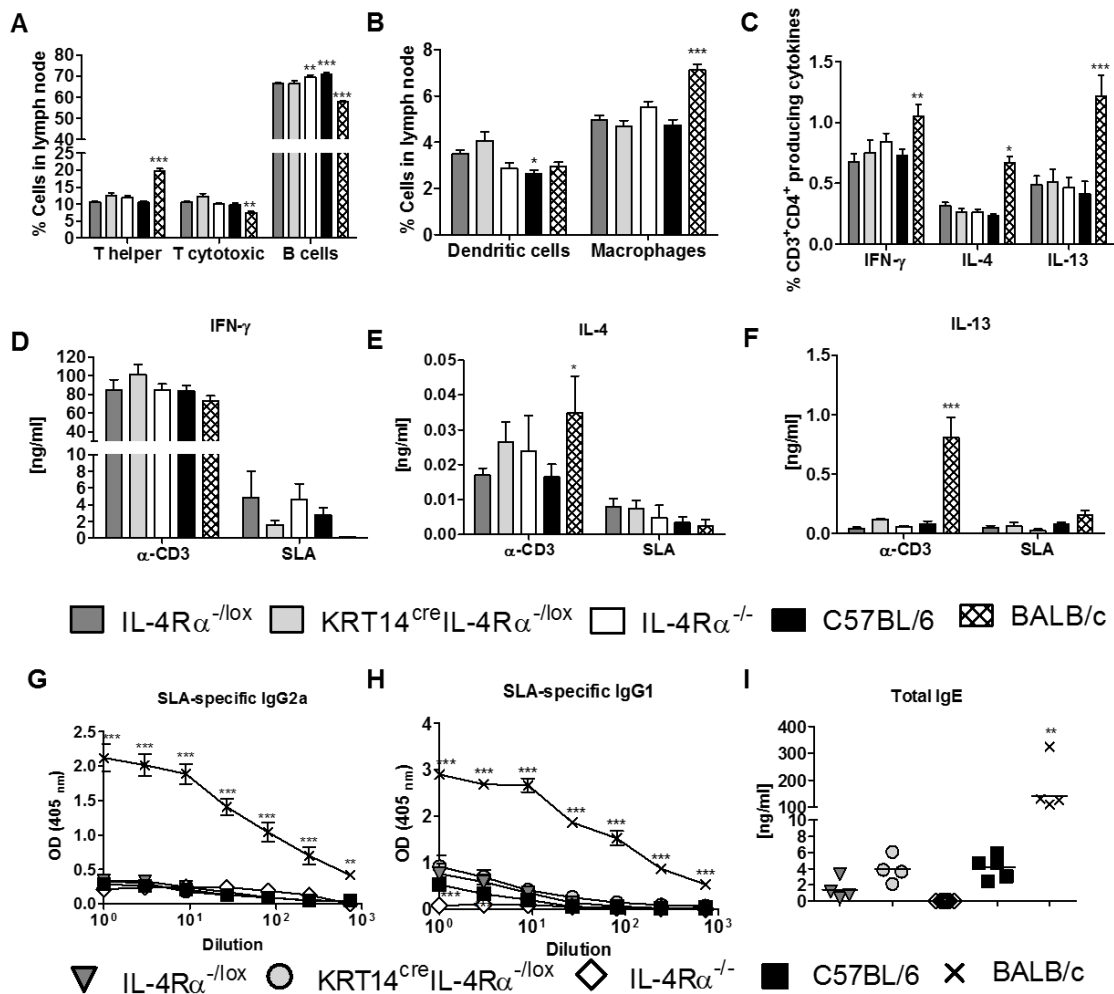


Figure 2.5 KRT14^{cre}IL-4R $\alpha^{-/-lox}$ C57BL/6 mice appear to develop a Th1 immune response similar to littermate control and resistant wild-type C57BL/6 mice during *L. major* LV39 infection in the footpad murine model. At 8 weeks post-infection, mice were sacrificed and popliteal draining lymph nodes retrieved. Single cell suspensions obtained from the lymph nodes were extracellularly stained for the following populations: (A) T helper (CD3⁺CD4⁺), T cytotoxic (CD3⁺CD8⁺), B cells (CD19⁺B220⁺); (B) dendritic cells (CD11c^{hi}MHCII^{hi}) and macrophages (CD11b^{hi}CD11c^{hi}MHCII^{hi}), and also (C) stimulated with PMA/ionomycin/monensin, followed by staining for intracellular cytokine production. Cells were re-stimulated with α -CD3 and soluble leishmanial antigen (SLA) for 72 hours, after which cytokine production was measured with ELISA for (D) IFN- γ , (E) IL-4 and (F) IL-13. (G) *L. major*-specific type 1 (IgG2a) and (H-I) type 2 antibodies (IgG1 and total IgE) were measured in sera from 6 week infected mice by ELISA. Data is representative of two experiments. Statistical analysis was performed with comparisons to the control IL-4R $\alpha^{-/-lox}$ littermate mice group, as significant (*, $p < 0.05$, **, $p < 0.01$; ***, $p < 0.001$).

2.4.6 Natural route of *L. major* infection in murine model does not dramatically alter the outcome of disease in KRT14^{cre}IL-4R α ^{-/lox} mice on both BALB/c and C57BL/6 genetic backgrounds.

While the footpad model for *L. major* infections in mice is well accepted, recent studies have suggested that the ear model may be a better model, providing a more natural mode of infection, as it mimics the events that occur during parasite inoculation by the sandfly [281, 282]. We investigated whether a change in site of infection would alter the phenotype and immune response of the KRT14^{cre}IL-4R α ^{-/lox}. Mice were intradermally infected with a low dose of 1×10^4 stationary phase metacyclic *L. major* IL-81 or *L. major* LV39 promastigotes, into the left ear. As with the footpad infection model, disease progression was monitored during infection period, and immune response evaluated after the infection period.

Ear swelling and disease progression of KRT14^{cre}IL-4R α ^{-/lox} BALB/c mice during *L. major* IL-81 infection was high, similar to littermate control mice (Figure 2.6A), corresponding to high parasite burdens in the ear, lymph node, and spleen (Figure 2.6A). IL-4R α ^{-/-} and C57BL/6 mice had significantly lower swelling compared to littermate control mice, with IL-4R α ^{-/-} mice appearing to be completely healed by week 8. In the ear, parasite burden was significantly lower in IL-4R α ^{-/-} and C57BL/6 mice compared to littermate control mice. C57BL/6 mice also showed lower parasite burdens in the draining lymph node and spleen in comparison to littermate control (Figure 2.6A). The swelling and parasite burdens in wild-type BALB/c mice were similar to littermate control mice. *L. major* LV39 infection of KRT14^{cre}IL-4R α ^{-/lox} BALB/c mice resulted in a swelling profile in the ear almost identical to that of the littermate control (Figure 2.6B). Interestingly, parasite burden in the ear was significantly lower in all mouse groups, including KRT14^{cre}IL-4R α ^{-/lox} BALB/c mice, when compared to littermate control mice (Figure 2.6B), which was unexpected, and reproducible in both LV39 experiments. Only IL-4R α ^{-/-} mice showed a significant difference to littermate control mice, in burden of parasite in the lymph node, with the remaining mouse groups having similar burdens (Figure 2.6B). No parasites were present in the spleens of any of the mouse groups, indicating no dissemination to the spleen (Figure 2.6B). Collectively, the data indicates that despite a change in site of infection, KRT14^{cre}IL-4R α ^{-/lox} BALB/c mice remain susceptible to *L. major*, and remain unaffected by the abrogated IL-4R α signalling on keratinocytes. Interestingly, with *L. major* LV39, parasite burdens at the site of infection in these mice were significantly decreased.

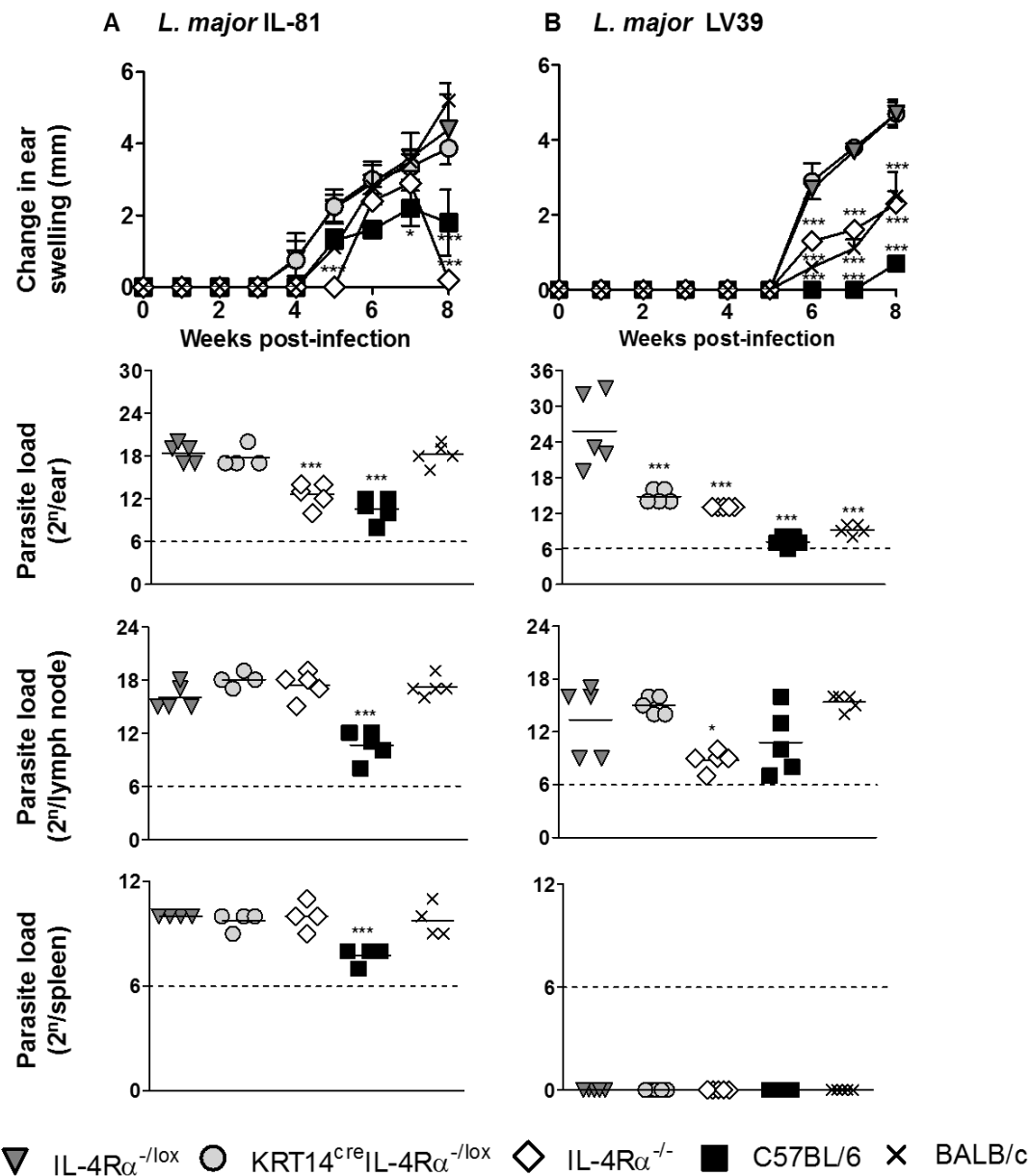


Figure 2.6 Cutaneous leishmaniasis is unaffected by the absence of IL-4R α signalling on keratinocytes on a BALB/c genetic background following low-dose *L. major* infection in the ear dermis. Mice were infected intra-dermally in the left ear, with 1×10^4 promastigotes of (A) *L. major* IL-81 or (B) *L. major* LV39. The change in ear swelling in mm was measured at weekly intervals (5-7 mice per group). Parasite burden was determined at week 8, by limiting dilution of homogenized ears, single-cell suspensions of the draining popliteal lymph nodes, and homogenised spleens. A representative of two individual experiments is shown with mean values \pm SEM. Statistical analysis was performed with comparisons to the control IL-4R α ^{-/lox} littermate mice group, as significant (***, $p < 0.001$).

Cellular immune response of KRT14^{cre}IL-4R α ^{-/lox} BALB/c mice in the lymph node remained unchanged, similar to littermate control mice, evident in the percentages of T helper, T cytotoxic, and B cells (Figure 2.7A), as well as dendritic cells and macrophages (Figure 2.7B),

8 weeks after an *L. major* IL-81 infection. Significant difference in cell population were only seen in T helper, responses, where IL-4R $\alpha^{-/-}$ mice showed higher levels, and C57BL/6 showed lower levels of these cells (Figure 2.7A). CD4⁺ T helper cytokine production (by intracellular staining of LN cells), and total cell cytokine production (by LN-re-stimulations), demonstrated similar levels of IFN- γ , IL-4 and IL-13 in KRT14^{cre}IL-4R $\alpha^{-/lox}$ BALB/c mice, similar to littermate control mice (Figure 2.7C-D). C57BL/6 mice appeared to produce significantly less CD4⁺ T helper IFN- γ and IL-13 as determined by intracellular staining of LN cells (Figure 2.7C). LN-re-stimulations with α -CD3 illustrated significantly lower IFN- γ in IL-4R $\alpha^{-/-}$ and C57BL/6 mice (Figure 2.7D), and significantly higher IL-4 in wild-type BALB/c mice (Figure 2.7E). No differences in antibody titres were observed in the sera of KRT14^{cre}IL-4R $\alpha^{-/lox}$ BALB/c mice compared to littermate control mice (Figure 2.7G-I). In comparison to littermate controls, IL-4R $\alpha^{-/-}$ mice had significantly higher levels of SLA-specific IgG2a (Figure 2.7G), lower levels of IgG1 (Figure 2.7H), and undetectable levels of IgE (Figure 2.7I). Significantly lower levels of IgG1 (Figure 2.7H), and undetectable levels of IgE (Figure 2.7I) was observed with C57BL/6 mice compared to littermate controls, after infection with *L. major* IL-81. Together, these results confirm that regardless of the site of infection, IL-4R α signalling on keratinocytes in BALB/c mice do not contribute to T helper responses during *L. major* IL-81, evident in unchanged cellular responses, cytokine production, and antibody titres.

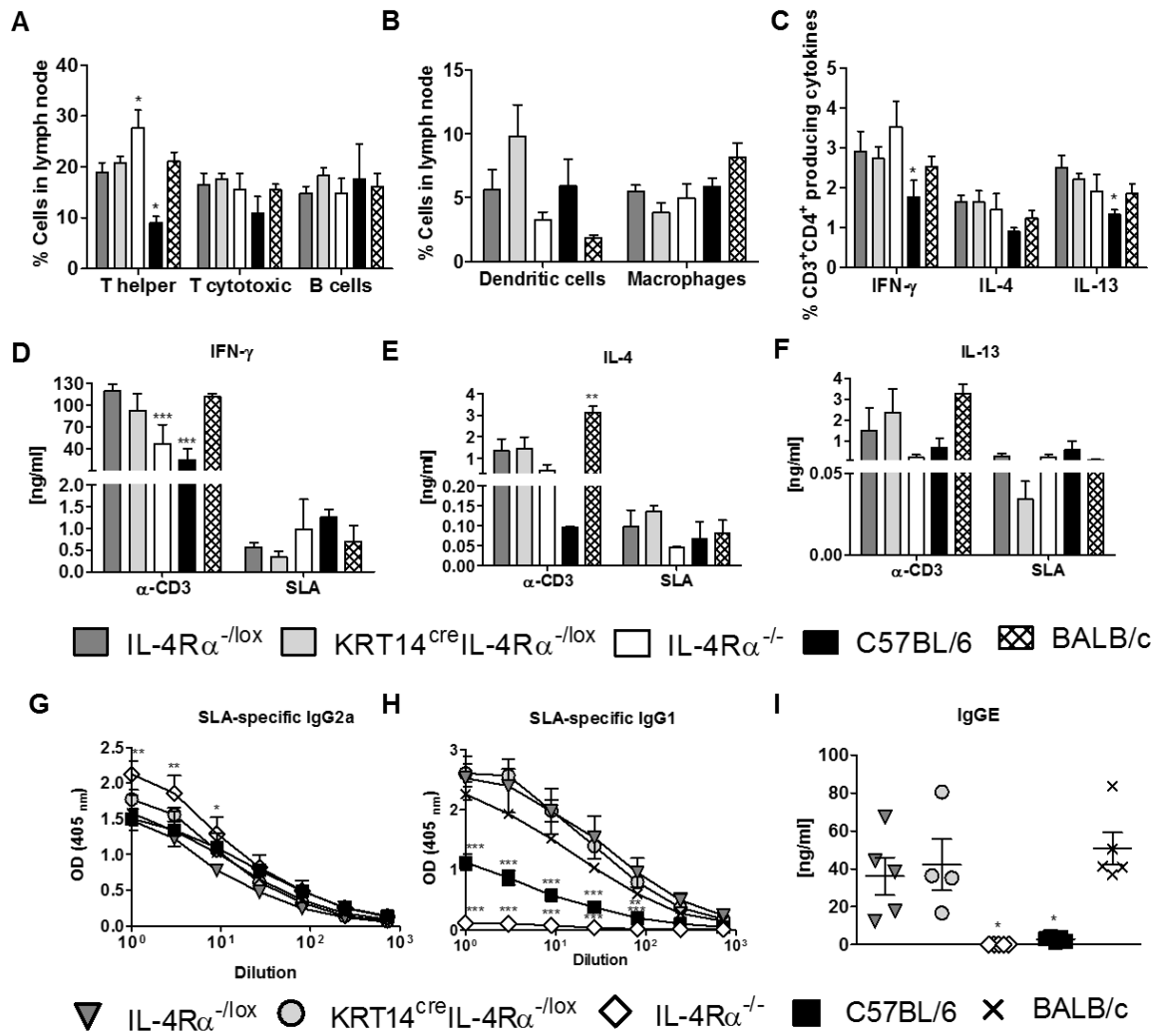


Figure 2.7 Low-dose infection in the ear dermis does not significantly alter immune response of KRT14^{cre}IL-4R $\alpha^{-/-}$ mice on a BALB/c genetic background, demonstrating a similar type 2 immune response when compared to littermate control mice during *L. major* IL-81 infection. At 8 weeks post-infection, mice were sacrificed and popliteal draining lymph nodes retrieved. Single cell suspensions obtained from the lymph nodes were extracellularly stained for the following populations: (A) T helper (CD3⁺CD4⁺), T cytotoxic (CD3⁺CD8⁺), B cells (CD19⁺B220⁺); (B) dendritic cells (CD11c^{hi}MHCII^{hi}) and macrophages (CD11b^{hi}CD11c^{lo}MHCII^{hi}), and also (C) stimulated with PMA/ionomycin/monensin, followed by staining for intracellular cytokine production. Cells were re-stimulated with α -CD3 and soluble leishmanial antigen (SLA) for 72 hours, after which cytokine production was measured with ELISA for (D) IFN- γ , (E) IL-4 and (F) IL-13. (G) *L. major*-specific type 1 (IgG2a) and (H-I) type 2 antibodies (IgG1 and total IgE) were measured in sera from 6 week infected mice by ELISA. Data is representative of two experiments. Statistical analysis was performed with comparisons to the control IL-4R $\alpha^{-/-}$ lox littermate mice group, as significant (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

After infection with *L. major* LV39, the cellular response of KRT14^{cre}IL-4R $\alpha^{-/-}$ BALB/c mice had remained unchanged, in comparison to littermate control mice (Figure 2.8A). This is depicted by the percentages of T helper, T cytotoxic, and B cells (Figure 2.8A), as well as dendritic cells and macrophages (Figure 2.8B) that remained unchanged in these mice.

Significant differences in T helper responses were observed in IL-4R α ^{-/-} and wild-type BALB/c mice showing higher levels of these cells, while C57BL/6 showed significantly higher levels of T cytotoxic cells (Figure 2.8A). CD4⁺ T helper cytokine production (by intracellular staining of LN cells) and total cell cytokine production (by LN-restimulations) demonstrated similar levels of IFN- γ , IL-4 and IL-13 in KRT14^{cre}IL-4R α ^{-/lox} BALB/c mice, similar to littermate control mice (Figure 2.8C-D). Intracellular staining of LN cells demonstrated significantly less IFN- γ , IL-4 and IL-13 produced by the CD4⁺ T helper of IL-4R α ^{-/-}, C57BL/6 and wild-type BALB/c mouse groups (Figure 2.8C), compared to littermate controls, further confirmed with ELISA detection of α -CD3-LN-re-stimulations (Figure 2.8D-E). In comparison to littermate controls, C57BL/6 mice appeared to produce significantly less IL-13 after LN-restimulations with α -CD3 and SLA (Figure 2.8F). IL-4R α ^{-/-} mice were also seen to have produced significantly less IL-13 after LN-restimulations with α -CD3 in comparison to littermate control mice (Figure 2.8F). No differences in antibody titres were observed in the sera of KRT14^{cre}IL-4R α ^{-/lox} BALB/c mice compared to littermate controls after *L. major* LV39 infection (Figure 2.8G-I). Wild-type BALB/c and C57BL/6 mice seemed to have significantly less titres of SLA-specific IgG2a (Figure 2.8G). Significantly lower levels of IgG1 were detected in IL-4R α ^{-/-} and C57BL/6 mice compared to littermate control mice (Figure 2.8H). Total IgE was shown to be significantly low in IL-4R α ^{-/-}, C57BL/6 and wild-type BALB/c mice in comparison to littermate control mice (Figure 2.8I). These findings confirm that during the ear infection, IL-4R α signalling on keratinocytes in BALB/c mice does not determine the dominant T helper response, during *L. major* LV39, evident in unchanged cellular responses, cytokine production, and antibody titres.

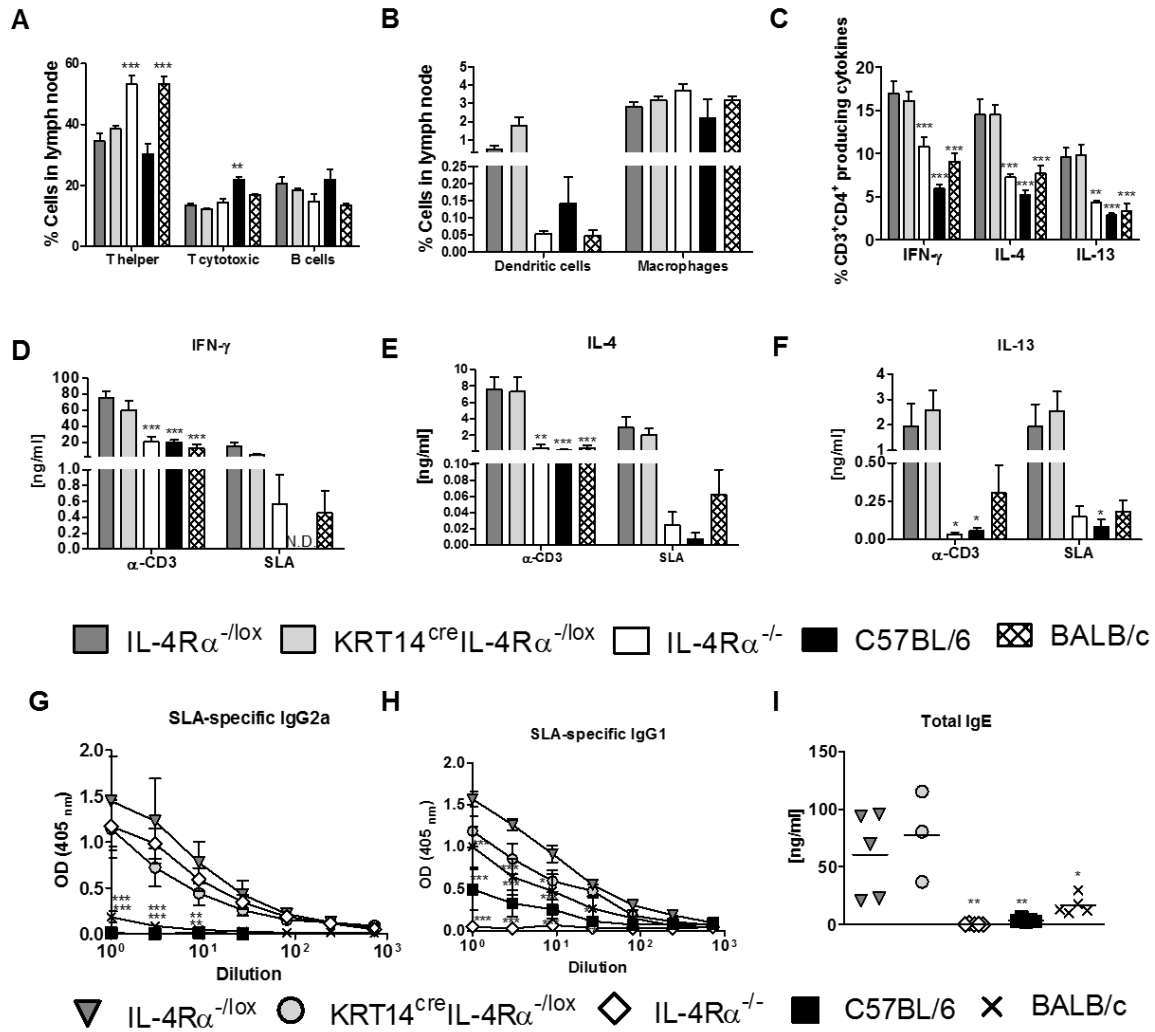


Figure 2.8 Immune response of KRT14^{cre}IL-4R $\alpha^{-/-}$ mice on a BALB/c genetic background remains unaffected with low-dose infection of less virulent *L. major* LV39 in the ear dermis. At 8 weeks post-infection, mice were sacrificed and popliteal draining lymph nodes retrieved. Single cell suspensions obtained from the lymph nodes were extracellularly stained for the following populations: (A) T helper (CD3⁺CD4⁺), T cytotoxic (CD3⁺CD8⁺), B cells (CD19⁺B220⁺); (B) dendritic cells (CD11c^{hi}MHCII^{hi}) and macrophages (CD11b^{hi}CD11c^{hi}MHCII^{hi}), and also (C) stimulated with PMA/ionomycin/monensin, followed by staining for intracellular cytokine production. Cells were re-stimulated with α -CD3 and soluble leishmanial antigen (SLA) for 72 hours, after which cytokine production was measured with ELISA for (D) IFN- γ , (E) IL-4 and (F) IL-13. (G) *L. major*-specific type 1 (IgG2a) and (H-I) type 2 antibodies (IgG1 and total IgE) were measured in sera from 6 week infected mice by ELISA. Data is representative of two experiments. Statistical analysis was performed with comparisons to the control IL-4R $\alpha^{-/-}$ littermate mice group, as significant (*, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$).

As with the footpad infection model, we tested the ear infection model using KRT14^{cre}IL-4R $\alpha^{-/-}$ mice on a C57BL/6 genetic background (Figures 2.9-11). Both *L. major* IL-81 and LV39 infections resulted in decreased swelling in KRT14^{cre}IL-4R $\alpha^{-/-}$ C57BL/6, mice IL-4R $\alpha^{-/-}$ and C57BL/6 mice, with significantly higher swelling observed in wild-type BALB/c mice when

compared to littermate control mice (Figure 2.9A-B). KRT14^{cre}IL-4R α ^{-lox} C57BL/6 had similar parasite burdens to littermate control mice. Ear parasite burdens after both infections were significantly lower in IL-4R α ^{-/-} and C57BL/6 mice, while wild-type BALB/c mice showed significantly more parasites after the *L. major* IL-81 infection (Figure 2.9A). Wild-type BALB/c mice also showed significantly more parasites in the draining lymph after infection with *L. major* IL-81 (Figure 2.9A) and *L. major* LV39 (Figure 2.9B), while IL-4R α ^{-/-} and C57BL/6 mice only showed significantly less parasites in this organ after infection with *L. major* IL-81 (Figure 2.9A). Parasite dissemination to the spleen did not occur in any of the mouse groups, except wild-type BALB/c, mice during either *L. major* IL-81 (Figure 2.9A) or *L. major* LV39 infection (Figure 2.9B). These results demonstrate that the resistant phenotype observed in C57BL/6 mice remains intact even in the absence of IL-4R α -responsive keratinocytes, regardless of the strain of *L. major* parasite, or route of infection.

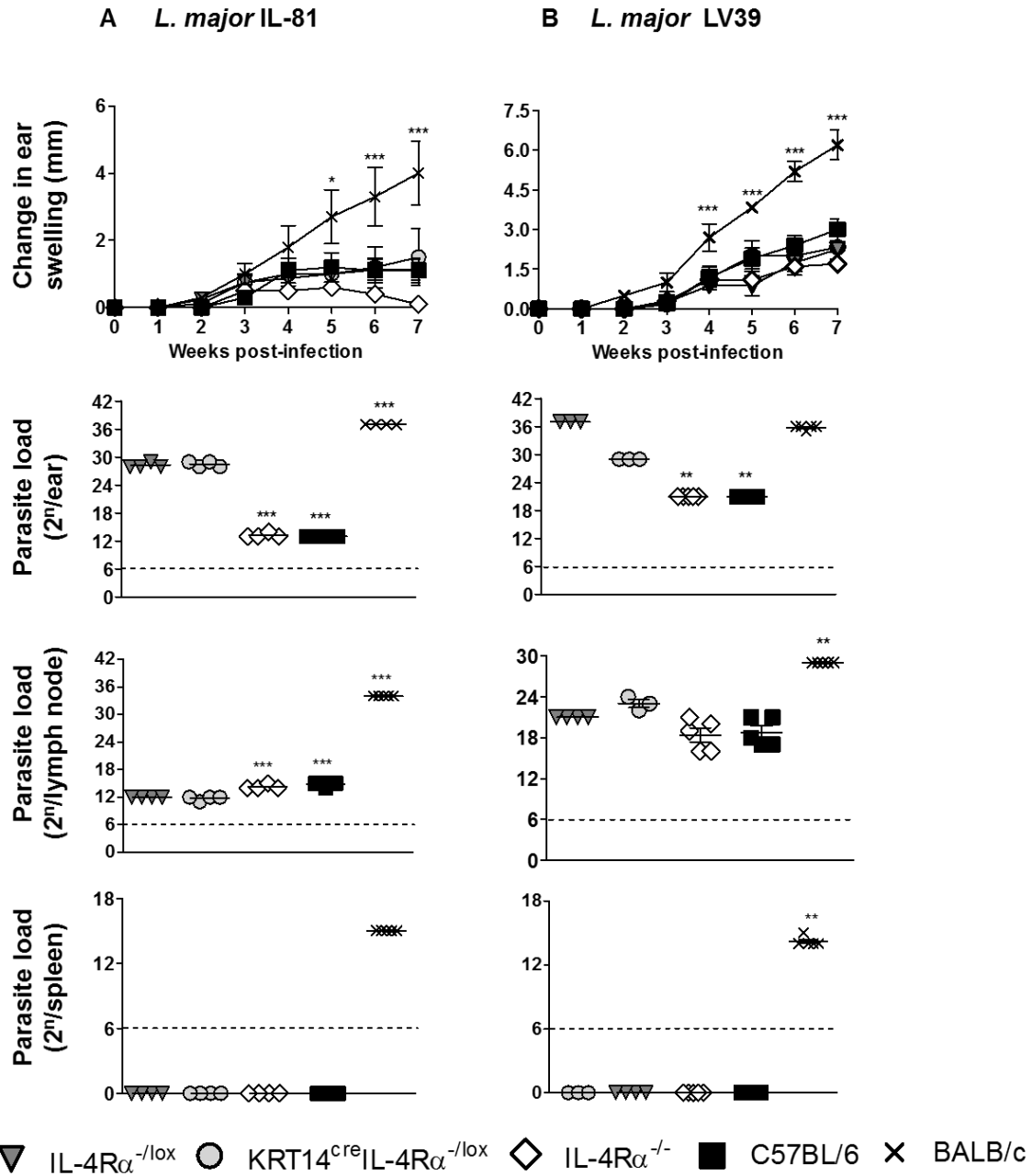


Figure 2.9 *L. major* phenotype is unaffected by the absence of IL-4R α signalling on keratinocytes on a C57BL/6 genetic background following low-dose *L. major* infection in the ear dermis. Mice were infected intra-dermally in the left ear, with 1×10^4 promastigotes of (A) *L. major* IL-81 or (B) *L. major* LV39. The change in ear swelling in mm was measured at weekly intervals (5-7 mice per group). Parasite burden was determined at week 8, by limiting dilution of homogenized ears, single-cell suspensions of the draining popliteal lymph nodes, and homogenised spleens. A representative of two individual experiments is shown with mean values \pm SEM. Statistical analysis was performed with comparisons to the control $IL-4R\alpha^{-/-}$ littermate mice group, as significant (***, $p < 0.001$).

After infection with *L. major* IL-81, only wild-type BALB/c mice were shown to produce significantly more T helper cells in the draining lymph node (Figure 2.10A). There were no significant differences in the levels of any of the infiltrating cell populations in the draining

lymph nodes of KRT14^{cre}IL-4R α ^{-lox} C57BL/6 mice when compared to littermate control mice (Figure 2.10A-B). Intracellular staining of LN cells demonstrated no significant differences in CD4⁺ T helper cytokine production of KRT14^{cre}IL-4R α ^{-lox} C57BL/6 mice as compared to littermate control mice (Figure 2.10C). C57BL/6 mice appeared to produce more CD4⁺ T helper IFN- γ , but was not significant, and wild-type BALB/c mice were seen to produce significantly more CD4⁺ T helper IL-4 (Figure 2.10C). Restimulation of LN cells for total cell cytokine production, demonstrated no significant differences in the cytokine production of KRT14^{cre}IL-4R α ^{-lox} C57BL/6 mice as compared to littermate control mice (Figure 2.10D-F). After LN re-stimulations with α -CD3, IL-4R α ^{-/-} and C57BL/6 mice produced significantly less IFN- γ (Figure 2.10D). Wild-type BALB/c mice were shown to produce significantly more IL-4 (Figure 2.10E) and IL-13 (Figure 2.10F) after α -CD3 LN re-stimulations. After infection with *L. major* IL-81, SLA-specific Type 1 (IgG2a) and type 2 (IgG1 and IgE) antibody titres in KRT14^{cre}IL-4R α ^{-lox} C57BL/6, IL-4R α ^{-/-}, C57BL/6 and littermate control mice showed similarly low to no levels of each antibody, with a highly significant increase in IgG2a, IgG1, and IgE observed in WT BALB/c mice (Figure 2.10G-I). Collectively, the data confirms observations made in the footpad model. During an ear infection with *L. major* IL-81, abrogation of IL-4R α -signalling on keratinocytes in C57BL/6 mice does not render these mice susceptible, as postulated. This was evident in control of ear swelling, cellular infiltration in the LN as well as cellular and humoral immunity.

Following *L. major* LV39 infection, extracellular staining on isolated draining lymph nodes showed no significant differences in cell populations of KRT14^{cre}IL-4R α ^{-lox} C57BL/6 mice (Figure 2.11A-B) compared to littermate controls, with IL-4R α ^{-/-} and wild-type BALB/c mice producing significantly more T helper cells and significantly less B cells (Figure 2.11A). Intracellular staining of LN cells showed no significant differences in CD4⁺ T helper cytokine production of KRT14^{cre}IL-4R α ^{-lox} C57BL/6 mice (Figure 2.11C) in comparison to littermate controls, while wild-type BALB/c mice produced significantly more CD4⁺ T helper IL-4 (Figure 2.11C). After LN re-stimulations with α -CD3 and SLA, total cell cytokine production of KRT14^{cre}IL-4R α ^{-lox} C57BL/6 LN cells remained similar to littermate control (Figure 2.11D-F), while IFN- γ was significantly decreased in IL-4R α ^{-/-} mice (Figure 2.11D), and IL-4 as well as IL-13 were significantly increased in wild-type BALB/c (Figure 2.11E-F). SLA-specific Type 1 (IgG2a) and type 2 (IgG1 and total IgE) antibody titres in KRT14^{cre}IL-4R α ^{-lox} C57BL/6, IL-4R α ^{-/-}, C57BL/6 and littermate control mice showed similarly low to no levels of each antibody, with a highly significant increase in IgG2a, IgG1, and total IgE observed in

wild-type BALB/c mice after *L. major* LV39 infection (Figure 2.11G-I). Together, these findings confirm, that in C57BL/6 mice, IL-4R α -responsive keratinocytes do not contribute to protective responses during *L. major* infection in the ear, as illustrated by unaltered cellular responses, cytokine production and humoral responses.

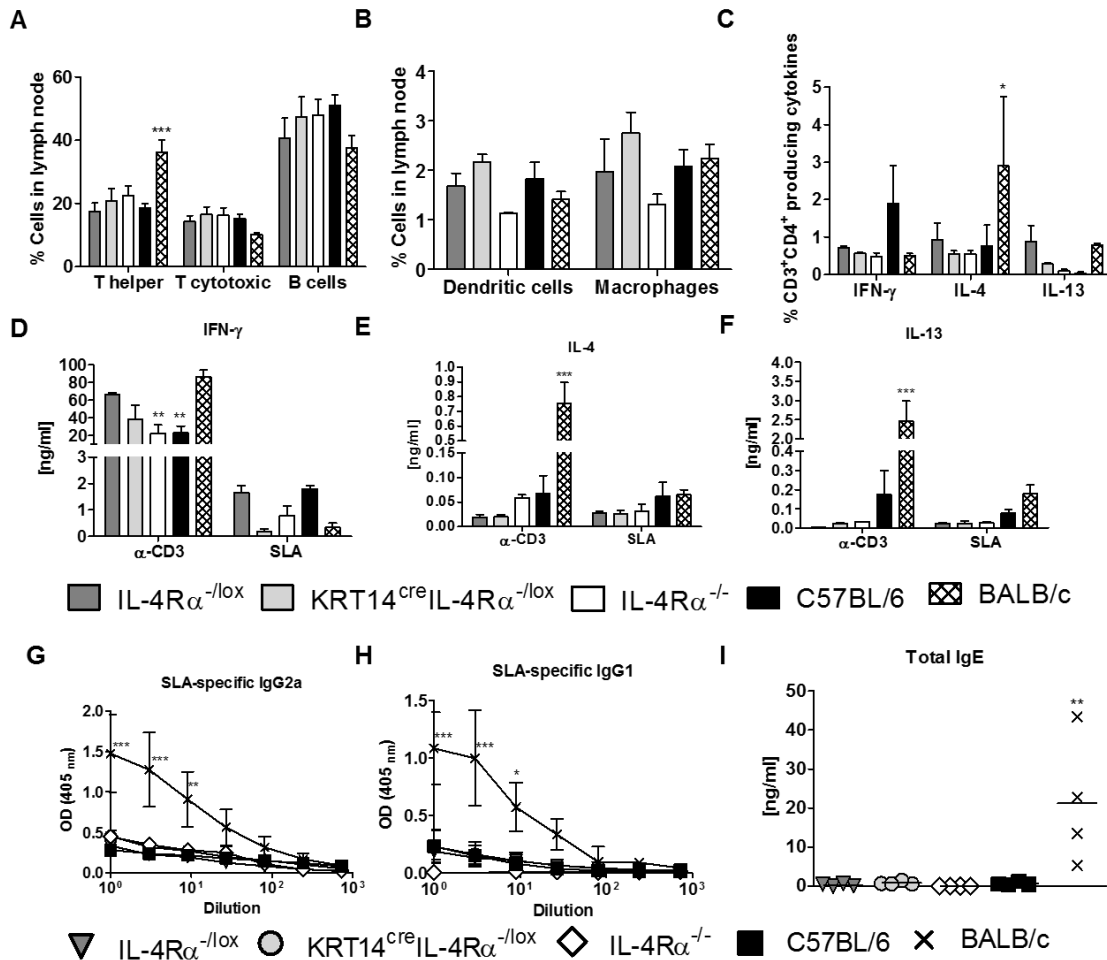


Figure 2.10 A similar type 1 immune response is observed in KRT14^{cre}IL-4R α ^{-lox} mice on a C57BL/6 genetic background following low-dose infection in the ear dermis during *L. major* IL-81 infection. At 8 weeks post-infection, mice were sacrificed and popliteal draining lymph nodes retrieved. Single cell suspensions obtained from the lymph nodes were extracellularly stained for the following populations: (A) T helper (CD3⁺CD4⁺), T cytotoxic (CD3⁺CD8⁺), B cells (CD19⁺B220⁺); (B) dendritic cells (CD11c^{hi}MHCII^{hi}) and macrophages (CD11b^{hi}CD11c-MHCII^{hi}), and also (C) stimulated with PMA/ionomycin/monensin, followed by staining for intracellular cytokine production. Cells were re-stimulated with α -CD3 and soluble leishmanial antigen (SLA) for 72 hours, after which cytokine production was measured with ELISA for (D) IFN- γ , (E) IL-4 and (F) IL-13. (G) *L. major*-specific type 1 (IgG2a) and (H-I) type 2 antibodies (IgG1 and IgE) were measured in sera from 6 week infected mice by ELISA. Data is representative of two experiments. Statistical analysis was performed with comparisons to the control IL-4R α ^{-lox} littermate mice group, as significant (*, p<0.05, **, p<0.01, ***, p<0.001).

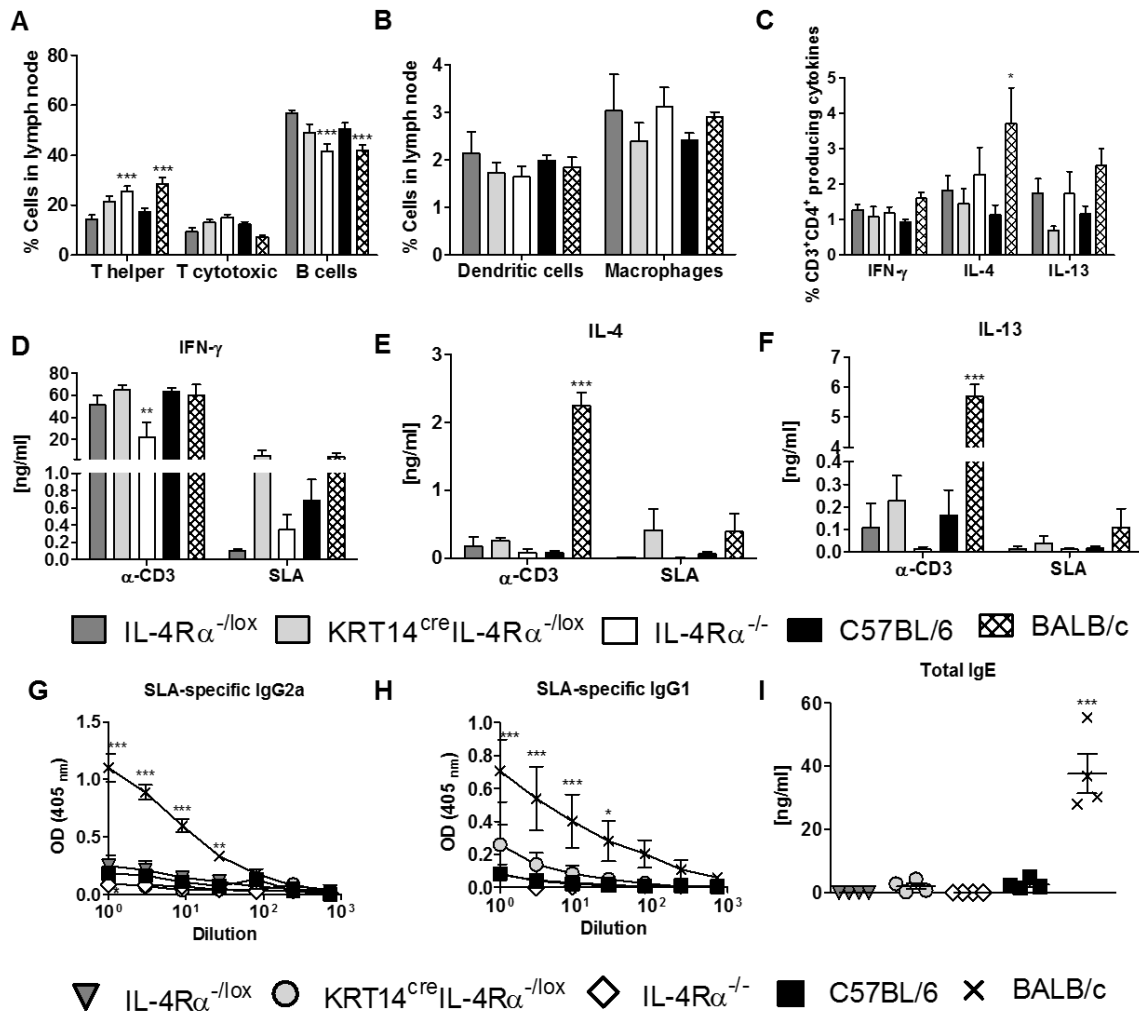


Figure 2.11 Lower virulence of *L. major* LV39 does not appear to alter the type 1 immune response observed in KRT14^{cre}IL-4Rα^{-/-lox} mice on a C57BL/6 genetic background similar to littermate control mice, following low-dose infection in the ear dermis. At 8 weeks post-infection, mice were sacrificed and popliteal draining lymph nodes retrieved. Single cell suspensions obtained from the lymph nodes were extracellularly stained for the following populations: (A) T helper (CD3+CD4+), T cytotoxic (CD3+CD8+), B cells (CD19+B220+); (B) dendritic cells (CD11chiMHCIIhi) and macrophages (CD11bhiCD11c-MHCIIhi), and also (C) stimulated with PMA/ionomycin/monensin, followed by staining for intracellular cytokine production. Cells were re-stimulated with α-CD3 and soluble leishmanial antigen (SLA) for 72 hours, after which cytokine production was measured with ELISA for (D) IFN-γ, (E) IL-4 and (F) IL-13. (G) *L. major*-specific type 1 (IgG2a) and (H-I) type 2 antibodies (IgG1 and total IgE) were measured in sera from 6 week infected mice by ELISA. Data is representative of two experiments. Statistical analysis was performed with comparisons to the control IL-4Rα^{-/-lox} littermate mice group, as significant (*, p<0.05, **, p<0.01; ***, p<0.001).

2.5 Discussion

L. major is the most studied strain of the *leishmania* parasites [293], and mouse models for this disease has been well studied, both in our laboratory [94, 132, 135, 294] as well as in other groups [285-287]. The outcome of *L. major* infection is dependent on the development of a

polarized Th1 or Th2 response that is associated with resistance or susceptibility, respectively [176]. IL-4 has long been considered as a characteristic Th2 driving cytokine in susceptible BALB/c mice during *L. major* infection [78, 176, 179, 182, 295, 296]. Kopf *et al.* showed that disrupting the IL-4 gene in BALB/c mice resulted in these generally susceptible mice, to become resistant to *L. major* infection [179]. In contrast, Stager *et al.* demonstrated that BALB/c mice with a deletion for IL-4 or IL-4R α were more susceptible during *L. donovani* infection compared to wild-type BALB/c mice, indicating a role for protection for IL-4, IL-13 and IL-4R α in protection within the *L. donovani* model. More recent studies have demonstrated a role for IL-4 in driving a polarised Th1 response, usually evident in genetically resistant C57BL/6 mice during infection with *L. major* [235, 291]. One of the pivotal studies was performed by Biedermann *et al.* through *in vitro* and *in vivo* experiments [235]. Here, the authors generated dendritic cells from bone-marrow cells they derived from the femurs and tibias of BALB/c mice, and incubated them with a synthetic immune-stimulatory oligomer (CpG DNA), in the presence or absence of recombinant IL-4 at a concentration of 10^3 U/ml. After IL-4 stimulation, they quantified the IL-12 present in the supernatant, using ELISA techniques, and found higher IL-12 production in the IL-4-treated sample. They next primed ovalbumin-specific CD4⁺ T cells with the previously CpG DNA+IL-4- or CpG DNA-IL-4-activated dendritic cells, and after a week, restimulated them with fresh APCs. IFN- γ was significantly upregulated when T cells were primed with dendritic cells that had been previously activated with CpG DNA and IL-4. Conversely, serum IFN- γ was significantly downregulated while IL-4 was upregulated when IL-4 was present during the priming of T cells. Together, these findings indicated that IL-4 could induce the production of IL-12 by APCs, and additionally, demonstrated that the Th2 characteristic IL-4 could in fact instruct dendritic cells to express a Th1-inducing phenotype [235]. Dendritic cells are the primary source of IL-12, and initiates antigen-specific immunity to *Leishmania* [212]. Biedermann *et al.* supplemented the *in vitro* findings data with *in vivo* mouse experiments, showing that mice treated with 1 μ g of recombinant IL-4 within the first 8 hours of infection, displayed significantly decreased swelling of the footpad and lesion, evident of a resistant phenotype. The authors found, however, that when they treated mice with a lower concentration of IL-4, or if treatment was much later, after 12 hours, it resulted in decreased production of IFN- γ , and an increase in the production of IL-4. Their findings suggested that early IL-4 was required to drive dendritic cells to elicit a Th1 response. From these observations, Biedermann *et al.* developed the principle of the “IL-4 instruction theory”. This study did, however, raise a question of the importance of biological quantities of IL-4, and the effect this had on the

immune response. Recently published work by Hurdal *et al.* from our laboratory [135], addressed this, using BALB/c mice with abrogated IL-4R α on dendritic cells, and illustrated that during *L. major* infection, these mice displayed hyper susceptibility to infection. This was evident in significantly increased swelling and parasite burdens, in addition to increased production of Th2 cytokines and type 2 antibodies. These findings further supported the findings reported by Biedermann *et al.* [235]. Following the theory, that early stage IL-4 is key in dendritic cell-driven Th1 immunity, Ehrchen *et al.* carried out experiments to further explore this, by focusing on the early stages of infection, looking at the initial infected site, specifically the microenvironment of the infected tissue [239]. The authors infected BALB/c and C57BL/6 mice in the footpad with *L. major* and screened for gene expression patterns using microarray analysis. In both mouse backgrounds, they found 189 genes that were significantly upregulated, and 16 genes that were down-regulated. For a more sensitive analysis, PCR was performed on skin samples, demonstrating an upregulation of chemokines, and the presence of cytokines (IL-12, TNF- α , IL-4, and IL-6), that were found to be more strongly induced in C57BL/6 mice compared to BALB/c mice [239]. Functional clustering indicated an overrepresentation of genes involved in inflammatory responses, chemotaxis, and cytokines, in the groups of genes that were found to be upregulated in both BALB/c and C57BL/6 mice, with some genes more highly regulated in the C57BL/6 mice. Additionally, genes that are involved in keratinocyte differentiation were also found to be overrepresented, suggesting an influence on their expression by the presence of *L. major* parasites. The authors next performed laser microdissection of the epidermal keratinocytes from skin, 16 hours after *L. major* infection, and applied the in-situ hybridization technique, followed by real-time PCR (RT-PCR). They showed a list of immune mediators that were induced, including IL-4, TNF α , IL-12 and IL-6, and illustrated cytokine induction by the skin and keratinocytes were higher in C57BL/6 mice. What this also meant, was that keratinocytes appeared to be a significant source of both early and particularly expressed genes in *L. major* infected footpads [239]. While Biedermann *et al.*, showed that exogenous recombinant IL-4 could drive dendritic cells to instruct a Th1 response [235], Ehrchen and his group set out to illustrate the role of local endogenous IL-4, by blocking the endogenous IL-4 with exogenous anti-IL-4 antibody. Here, the authors carried out *L. major* infections in C57BL/6 mice, where, at the time of infection with *L. major* parasites into the footpad, 1 mg neutralizing rat anti-mouse IL-4 antibody or irrelevant rat IgG neutralizing was also injected, followed by a second treatment with antibody 4 hours later. A week later, analysis of *L. major* antigen-specific CD4⁺ cytokine secretion illustrated significantly increased

quantities of IL-4 and IL-13, and significantly decreased IFN- γ . This indicated a switch in these genetically resistant C57BL/6 mice to a Th2 phenotype [235].

Essentially, two important ideas are introduced. Firstly, keratinocytes appear to be highly activated by *L. major* parasites, and in response, secrete chemokines and cytokines, one of which is IL-4, which is upregulated in genetically resistant C57BL/6 mice [239]. Secondly, IL-4 can instruct dendritic cells to produce Th1 cytokines and cause a shift towards a Th1 immune response [235, 239]. As mentioned previously, in addition to IL-4, IL-13 also signals via IL-4R α [98]. Most studies have shown that IL-13 plays a role in susceptibility to *L. major* [291, 297], and *L. mexicana* infection [298]. Mohrs *et al.*, however, showed that IL-13 can have a protective role during chronic cutaneous leishmaniasis, and this is dependent on signalling via the IL-13 receptor [233]. During the study by Ehrchen *et al.*, IL-13 expression was not found to be significant factor. Keratinocytes can, however, signal and secrete IL-13 [64, 67]. Following on from the studies by Biedermann *et al.* [235] and Ehrchen *et al.* [239], we aimed to investigate whether IL-4R α on keratinocytes, and the IL-4/IL-13 signalling via this receptor, plays a role in disease outcome during cutaneous leishmaniasis. If IL-4R α is indeed demonstrated to be important for disease outcome, this would suggest that the IL-4/IL-13 released by these keratinocytes could signal and influence keratinocytes, in an autocrine manner, or influence dendritic cells in a paracrine manner. The autocrine/paracrine tendency of keratinocytes has been illustrated during atopic dermatitis [299]. Here, Yoon *et al.* showed that endogenous toll-like receptor 4 induced keratinocytes to secrete IL-23, which, in a paracrine manner, influences dendritic cells to drive production of IL-22 [299], which is important for antibacterial functions [300-302], keratinocyte proliferation (autocrine) and epidermal thickening [303, 304]. To address our abovementioned hypothesis, our laboratory generated a keratinocyte-specific IL-4R α knock-out mouse model using the Cre/*loxP* system under the control of the K14 promotor. The use of these mice in *L. major* infection models, in addition to cell based assays, allowed us to evaluate disease progression and outcome, in the absence of IL-4R α -responsive keratinocytes.

The footpad mouse model in *L. major*, is well accepted and widely used [94, 132, 135, 233, 284, 305-307], as the clinical progression of disease can be evaluated by simply measuring the swelling of the footpad, and the subcutaneous route of infection causes a more potent immune response, compared to intradermal infections [308]. Additionally, when the subcutaneous route was compared to an intradermal route, during *L. donovani* infection in BALB/c mice, the subcutaneous inoculation elicited a more potent Th1 responses [309]. Infection of KRT14^{cre}IL-4R α ^{-lox} BALB/c mice in the footpad with *L. major* IL-81 or LV39 revealed no role for IL-4R α -

responsive keratinocytes in modulating disease outcome. KRT14^{cre}IL-4R α ^{-/lox} BALB/c mice presented as susceptible mice, as evident in increased swelling and parasite burdens, which is characteristic of genetically susceptible BALB/c mice [293]. While global IL-4R α ^{-/-} BALB/c mice were resistant to *L. major* IL-81 as shown by Hurdayal *et al.* and Radwanska *et al.* [310], these mice appeared to be susceptible to *L. major* LV39 infection, contradictory to observations made by previous studies in the laboratory [94, 132, 135, 233, 234] with this strain. This contradictory finding is, however, not entirely unusual, as susceptibility to *L. major* LV39 by global IL-4R α ^{-/-} BALB/c has been demonstrated in early studies by Noben-Trauth *et al.* [291]. This difference in disease outcome observed here is due to the difference in the source and sub strain of the *L. major* LV39. While the infections in mice on BALB/c genetic background, in the footpad model in our study used the LV39 sub strain sourced from ATTC, subsequent infections used the LV39 sub strain sourced from the University of Lausanne. Additionally, Hurdayal *et al.* and Radwanska *et al.* [132, 135] used sub strains from Mattner *et al.* [180]. Furthermore, Kebaier *et al.* also demonstrated that different isolates of *L. major* parasite strains can differ in virulence, thus varying the influence the strain has on the course of disease it elicits in mouse models [288]. KRT14^{cre}IL-4R α ^{-/lox} BALB/c showed dissemination of parasites to the spleen similar to littermate control mice, similar to wild-type BALB/c mice, after *L. major* infection, which is expected in susceptible wild-type BALB/c mice [182, 311]. In these mice, the mounting of a Th2 response, along with alternative activation of macrophages, is the reason why parasites disseminate and cause severe disease [211, 312-314]. The parasites can use the polyamines generated from alternative activation for their growth and survival [135]. The presence of *L. major* parasites in the spleens of global IL-4R α ^{-/-} BALB/c and C57BL/6 is not entirely unexpected, as patchwork mutation (PWK) mice, that are self-healing, and able to control parasite multiplication, have demonstrated visceral dissemination of the parasites [312]. Disease progression in BALB/c mice infected with *L. major* corresponds to an upregulation Th2 cytokines, CD4⁺ Th2 cells, and type 2 antibody responses [132, 135, 234, 295, 297, 315]. Resistant C57BL/6 had significantly less T helper cells. CD4⁺ T helper and CD8⁺ T cytotoxic responses are important for an effective Th1 immune response to be mounted as well as resolution of the disease [174, 293, 316]. C57BL/6 mice appeared to have decreased CD4⁺ Th cells, which may have been due to the evaluation being made at the end of the infection period, when initial immune response have already been established. Increased infiltration of macrophages to the draining lymph node in C57BL/6 mice, could be linked to the roles they play in protection, being important not just for uptake of parasites and establishment of an immune response, but also for their killing function by classically activated macrophages [174,

177, 205, 206, 237]. Our intracellular staining panel did not include markers for iNOS and Arg, to indicate the presence of classically or alternatively activated macrophages in the mouse groups, however, we could assume KRT14^{cre}IL-4R α ^{-lox} BALB/c mice may have shown higher frequencies of alternatively activated macrophages, as they were susceptible to *L. major* infection [134, 208, 315, 317]. CD4⁺ T cell production of cytokines, illustrated both Th1 (IFN- γ) and Th2 cytokines (IL-4 and IL-13) in KRT14^{cre}IL-4R α ^{-lox} BALB/c mice, instead of a polarised Th2 response as expected of susceptible wild-type BALB/c mice [176, 318]. This phenomenon of a mixed response has been observed in wild-type BALB/c mice infected with *L. infantum* [319]. C57BL/6 demonstrated a significant upregulation of IFN- γ , while IL-4 and IL-13 were significantly downregulated, indicative of a clear Th1 polarisation for a resistant phenotype [174, 176, 209, 318]. Clinically resistant global IL-4R α ^{-/-} BALB/c mice also showed a downregulation of IL-13, similar to recent publications [94, 132, 135, 234, 320]. Compared to intracellular staining, which measures only CD4⁺ T cell production of cytokines, ELISA evaluation provides an indication of total cytokine production by total lymph node cells. KRT14^{cre}IL-4R α ^{-lox} BALB/c mice produced both Th1 (IFN- γ) and Th2 cytokines (IL-4 and IL-13) at similar levels, similar to above. Despite their healing phenotype, C57BL/6 mice had downregulated IFN- γ produced by various lymph node cells and significantly lower quantities of IL-4 and IL-13. Similarly, Anderson and colleagues recently showed that while C57BL/6 infected with *L. major* mounted a Th1 polarised immune response as expected, these mice did not heal their lesions [321]. This highlights the fact that the level of IFN- γ -secreting cells does not necessarily, or in all cases, reflect a healing response. Resistant global IL-4R α ^{-/-} BALB/c mice also had downregulated levels of IL-4 and IL-13 similar to previous studies [135, 233]. KRT14^{cre}IL-4R α ^{-lox} BALB/c produced Type 1 and 2 antibodies. Susceptible strains such as wild-type BALB/c mice, have been shown in early studies to produce lower levels of IgG2a and higher levels of the Type 2 antibodies IgG1 and total IgE [233, 322]. IgG1 is linked to progressive leishmaniasis, with this antibody being associated with an increase in lesion thickness in WT BALB/c mice [323]. While KRT14^{cre}IL-4R α ^{-lox} BALB/c mice did develop progressive swelling and lesions, these mice had fairly low levels of IgG1. Global IL-4R α ^{-/-} BALB/c and C57BL/6 mice showed extremely low to no levels of IgG1 and IgE, indicative of their inability to class switch. Together, the data suggested that BALB/c mice with abrogated IL-4R α were susceptible to *L. major* infection and did not develop a polarised T helper response. Furthermore, the data indicated that in BALB/c mice, IL-4R α on keratinocytes did not play a role in protection.

As mentioned earlier, previous studies suggest keratinocytes as the source of IL-4 to instruct dendritic cells to mount a Th1 polarised response [239]. Ehrchen *et al.* [239] showed that genetically resistant C57BL/6 mice appear to have significantly high levels of IL-4 secreted by keratinocytes, and the early local neutralization of the IL-4 resulted in susceptibility to *L. major* infection. We therefore tested our hypothesis using the KRT14^{cre}IL-4Rα^{-lox} on a C57BL/6 genetic background. For these experiments, IL-4Rα^{-/-} was also on a C57BL/6 genetic background, which is, to our knowledge, the first time the use of these mice has been reported. KRT14^{cre}IL-4Rα^{-lox} C57BL/6 mice behaved like littermate control mice, developing low swelling, and healed lesions by week 8 of *L. major* LV39 infection. During these *L. major* LV39 infections, KRT14^{cre}IL-4Rα^{-lox} C57BL/6 mice showed low parasite loads in the footpad, draining lymph node and spleen, similar levels of infiltrating cells, and the predominant production of IFN-γ, with lesser quantities of IL-4 and IL-13, similar to littermate controls (on the same C57BL/6 background). Antibody levels were unchanged, with low levels of type 1 and type 2 antibodies observed, corresponding to antibody profiles of C57BL/6 mice in previous studies [135, 233]. Interestingly, during both *L. major* IL-81 and LV39 infections, IL-4Rα^{-/-} C57BL/6 mice completely healed at the end of the infection period. These mice also showed significantly higher levels of B cells similar to C57BL/6 mice with both strains of *L. major*, which could not be explained. B cells have been shown to contribute to susceptibility to *L. major* infection [232, 324, 325]. Additionally, a recent study from our laboratory has indicated a role for IL-4Rα-responsive-B cells in susceptibility to *L. major* infection (Unpublished). Essentially, KRT14^{cre}IL-4Rα^{-lox} C57BL/6 were resistant, as expected of a C57BL/6 mouse. Together, these results suggested that if early IL-4 is instructing resistance to *L. major* infection as previously demonstrated [135, 235], it did not appear that the early IL-4 was in fact signalling through the IL-4Rα on keratinocytes.

An alternative to the subcutaneous footpad infection model, is an intradermal inoculation of low dose metacyclic promastigote *L. major* parasites (10-1000 parasites) in the ear, a site of infection that is becoming more favoured, as it better mimics natural infection by the sandfly, and is better at reproducing the natural lesion formed [281-283, 326-329]. KRT14^{cre}IL-4Rα^{-lox} BALB/c mice behaved similar to littermate controls, seen in swelling progression and parasite burden. During *L. major* LV39 infection, these mice showed significantly less parasites at the site of infection (the ear), similar to observations by Parihar *et al.* [283]. This suggests that these mice were able to control parasite replication in the ear. This could be attributed to iNOS-secreting macrophages and recently described, iNOS-secreting dendritic cells, shown to control intracellular parasitism [315, 330]. Global IL-4Rα^{-/-} BALB/c mice completely healed lesions

caused by *L. major* IL-81 and had increased swelling progression during *L. major* LV39 as seen above. Dissemination of parasites was seen in all mouse groups, only after *L. major* IL-81 infection. This strain is highly virulent, and as mentioned above, visceral dissemination of *L. major* parasites has been shown [135, 312]. Cellular and humoral responses of KRT14^{cre}IL-4Rα^{-lox} BALB/c mice after infection with *L. major* IL-81, remained unchanged similar to littermate controls, as above. As observed in the footpad infection model, global IL-4Rα^{-/-} BALB/c mice had upregulated T helper responses, while C57BL/6 had downregulated T helper cells. Both C57BL/6 and IL-4Rα^{-/-} BALB/c mice had decreased IFN-γ, similar to findings with the footpad infection model. As expected C57BL/6 mice showed decreased IL-13, while wild-type BALB/c mice had increased IL-4. Antibody responses of IL-4Rα^{-/-} BALB/c and C57BL/6 mice correlated with the expected antibody profile of a resistant mouse. Infection with *L. major* LV39 saw no change in immune response of KRT14^{cre}IL-4Rα^{-lox} BALB/c mice. Together, our data suggested that while IL-4Rα-responsive keratinocytes were not contributing to the overall T helper immune responses of KRT14^{cre}IL-4Rα^{-lox} BALB/c mice in the ear infection model, parasite replication at the site of infection appeared to be more controlled in these mice, in comparison to littermate controls. KRT14^{cre}IL-4Rα^{-lox} C57BL/6 mice infected with *L. major* had swelling and disease similar to littermate controls. With these experiments, global IL-4Rα^{-/-} C57BL/6 mice completely healed their lesions during *L. major* IL-81, as above, also showed resistance to *L. major* LV39, similar to C57BL/6 mice. This, again, highlights the importance of the source of the sub-strain, as explained above. Wild-type BALB/c mice had upregulated T helper cells, IL-4, and IL-13 after infection with *L. major* IL-81, as expected. KRT14^{cre}IL-4Rα^{-lox} C57BL/6 infected with *L. major* produced both Th1 and Th2 cytokines, however, unlike KRT14^{cre}IL-4Rα^{-lox} BALB/c mice, these mice did appear to have a more polarised Th1 cytokine production as expected of this genetic background. Together, these results indicate that IL-4Rα signalling on keratinocytes in C57BL/6 mice is not required for protection against *L. major* infection in the ear.

It would seem that the IL-4Rα-responsive keratinocytes may not be contributing to the early-IL-4-driven polarised Th1 response as suggested by Ehrchen *et al.* [239]. The question then remains, as to which of the many cell types that signal and secrete IL-4/IL-13 (highlighted in Chapter 1), could be contributing to this shift in the response during *L. major* infection. Neutrophils are the first cells to be recruited to the infection site, and can signal IL-4/IL-13 through the IL-4Rα [331]. While some studies have indicated a role for neutrophils in susceptibility to *L. major* infection [196, 237], others have shown their contribution to resistance to *L. major* [332, 333]. Alternatively, mast cells, that are also capable of signalling

and secreting IL-4/IL-13 through IL-4R α [334], could be the determining factor in the early-IL-4-driven polarised immune response to *L. major*. Mast cells can be found in the skin layer and contribute to the hosts' defence mechanisms [335]. Furthermore, as with neutrophils, they are one of the first cell types to have contact with the *Leishmania* parasite during infection [336]. These cells are also capable of producing nitric oxide [337], in addition to pro-inflammatory molecules that are stored within the granules found in the cytoplasm, and can be released within minutes of activation [338]. Mast cells release cytokines, including Th1 IFN- γ and TNF- α , and Th2 IL-4 and IL-10, during *Leishmania* infection, which can influence the immune response [339]. While degranulation of mast cells has been shown to contribute to susceptibility to *L. major* infection [340], Mauer *et al.* demonstrated a role for these cells in protection against *L. major* infection [341]. Using mast cell-deficient Kit(W)/Kit(W-v), Mauer *et al.* showed that during *L. major* infection, these mice developed significantly larger skin lesions than control mice, and when these mice were cutaneously reconstituted with mast cells, it normalized the development of lesions [341]. Furthermore, these Kit(W)/Kit(W-v) mice had significantly higher parasite burdens showed dissemination of parasites to the spleen, in comparison to control mice. Additionally, the recruitment of neutrophils and macrophages appeared to be dependent on the mast cells. Importantly, the recruitment of dendritic cells seemed to be heavily dependent on mast cells [341]. These findings provide sufficient evidence to suggest a possible role for mast cells in contributing to the IL-4-driven dendritic cell Th1 polarised response seen by Ehrchen *et al.* [239].

2.6 Conclusion

Our results illustrate that the abrogation of IL-4/IL-13 signalling via IL-4R α on keratinocytes results in a non-polarised immune response in BALB/c mice, but does not affect their non-healing phenotype. In these mice, however, the absence of IL-4R α -responsive keratinocytes does contribute to parasite control at the site of infection, in the ear model. We showed that the loss of IL-4/IL-13, signalling via IL-4R α on keratinocytes in C57BL/6 mice, does not alter the overall polarised Th1 response, or the healing phenotype. We have also shown, as have others, that a difference sub-strains/sources of *L. major* parasite can alter the immune response differently. Lastly, we have reported immune responses in global IL-4R α ^{-/-} C57BL/6 mice, during *L. major* infection for the first time. These mice remain resistant to infection, and compared to IL-4R α ^{-/-} BALB/c mice, have dampened antibody responses.

Chapter 3 IL-4R α –responsive keratinocytes are not required for control during acute schistosomiasis in mice

3.1 Introduction

Schistosomiasis is a neglected tropical parasite disease caused by blood flukes, the trematode flatworms, belonging to the *Schistosoma* genus. The disease affects approximately 200 million people each year, and is endemic in 52 countries across the globe [240, 241]. The life cycle of *Schistosoma mansoni* (*S. mansoni*) begins when infective cercariae penetrate the skin of the host and transform into schistosomula that migrate to peripheral vessels [342, 343]. Schistosomula mature to adult worms and lay eggs in the mesenteric venules. Some of the eggs are excreted in faeces, while others are shunted via the hepatic portal vein into the liver where they become trapped. The trapped eggs induce a T helper 2 (Th2) immune response that drives granuloma formation and tissue fibrosis, the key pathological features of schistosomiasis [134, 265, 266]. While there have been studies showing that cercariae can affect the function of dermal and epidermal cells [344–346], there have been very few studies investigating epidermal keratinocytes and their role during schistosomiasis [278, 279].

Keratinocytes make up a large part of the epidermal cellular population. These cells contribute to epidermal cytokine production, which influences the migration of inflammatory cells and can have systemic effects on the immune system [67]. The importance of epidermal keratinocytes as a physical barrier is well known [39]. However, their role in immunological defence is poorly understood. Recent studies have shown that keratinocytes can produce growth factors such as granulocyte colony-stimulating factor (G-CSF) and granulocyte macrophage colony-stimulating factor (GM-CSF), and cytokines such as tumour necrosis factor alpha (TNF- α); IL-3; IL-6 and IL-8 [53, 55, 59, 60, 67, 347]. Moreover, keratinocytes have been shown to play a key role in skin-related diseases such as atopic dermatitis and psoriasis, where these cells contribute to pathogenesis [54, 348, 349].

Murine models of schistosomiasis have played an important role in providing a better understanding of the elicited host immune responses, and defined a host-protective role for signalling through the IL-4 receptor α chain (IL-4R α) [130, 134, 136, 277]. A recent study by Ehrchen *et al.* postulated that IL-4 from keratinocytes drives a Th1 immune responses during *Leishmania major* (*L. major*) infection [239]. Considering the protective role that IL-4/IL-13 and IL-4R α play during acute schistosomiasis, we postulated that IL-4R α -responsive keratinocytes are crucial for host protection during *S. mansoni* infection.

In order to investigate the role of IL-4R α -responsive keratinocytes, we generated a keratinocyte-specific IL-4R α -deficient BALB/c mouse (KRT14^{cre}IL4R α ^{-lox}), using the Cre/loxP recombination system. These mice were infected with *S. mansoni* cercariae to evaluate their survival, organ pathology, and the immune responses elicited. We found that KRT14^{cre}IL4R α ^{-lox} mice were susceptible to acute schistosomiasis, similar to littermate controls. KRT14^{cre}IL4R α ^{-lox} mice developed sufficient Th2 immune responses as seen by T helper cytokines, type 2 antibodies, and B cell cytokines. Together, our findings suggest that IL-4R α -responsive keratinocytes are not required for control of acute schistosomiasis in mice.

3.2 Materials

All general reagents were purchased from either of the following suppliers: Merck Chemicals (Pty) Ltd.; Gibco Life Technologies; Invitrogen Life Technologies and Sigma Aldrich. Molecular biology reagents were purchased from ThermoScientific, BD Biosciences and Fermentas Life Sciences. Buffers and reagents are listed in the Appendix.

3.3 Methods

3.3.1 Ethical statement

All mouse experiments and protocols were performed in strict accordance with the South African national guidelines, as well as the Animal Research Ethics Committee of the Faculty of Health Sciences, University of Cape Town (license no. 014/003). All effort were made to minimise and reduce suffering of animals.

3.3.2 Generation of KRT14^{cre}IL-4R α ^{-lox} mice

Keratinocyte cell-specific IL-4R α -deficient (KRT14^{cre}IL4R α ^{-lox}) BALB/c mice were generated using the Cre/loxP system, and characterized in our laboratory (Supplementary Figure). Briefly, KRT14^{cre} mice (Jackson laboratory) were crossed with IL-4R α ^{-/-} BALB/c mice [233] and transgenic IL-4R α ^{lox/lox} mice [134] to generate hemizygous KRT14^{cre}IL-4R α ^{-lox} BALB/c mice. All mice were kept under specific pathogen-free conditions in individually ventilated cages. Experimental mice were age and sex matched.

3.3.3 Live *S. mansoni* infection

Mice were percutaneously infected with 100 live cercariae (7 week acute infection) or with 80 live cercariae (12 week mortality study). Snails were provided by the Schistosome Research Reagent Resource Center for distribution by BEI Resources, NIAID, NIH: *Schistosoma mansoni*, Strain NMRI Exposed *Biomphalaria glabrata*, Strain NMRI, NR-21962.

3.3.4 Isolation and *ex vivo* stimulation of draining lymph node cells

Single cell suspensions were prepared by pressing the draining mesenteric lymph nodes (MLN) through cell-strainers (70 μ M followed by 40 μ M). Cells were re-suspended in complete IMDM supplemented with 10% FCS and an antibiotic mix (penicillin and streptomycin at 100 U/ml each). Cells were cultured at 2×10^6 cells/ml in 96-well NUNC® plates coated with α -CD3 at 20 μ g/ml or in the presence of SEA (20 μ g/ml) and incubated at 37°C in a humidified incubator containing 5% CO₂. Supernatants were collected after 72 h and cytokines were measured by ELISA. The following cytokines were measured by sandwich ELISA: IL-4, IL-5, IL-6, IL-10, IL-13, IFN- γ and IL-17, as previously described [233]

3.3.5 Flow cytometry

MLN cells from *S. mansoni* infected mice were prepared as previously described (Section 3.3.4) and seeded (1×10^6 cells per well) for extracellular staining to analyse cellular populations. Briefly, cells were stained with 50 μ l antibody mix for 30 min at 4°C. After incubation, 150 μ l FACS buffer was added and cells were centrifuged at $5\,000 \times g$ for 5 min at 4°C. The pellet was resuspended in 200 μ l FACS buffer. The following panel was set up to stain for CD4⁺ T: CD3-Alexa700; CD4-PerCP; CD44-FITC; CD62L-APC. The B cell panel included: B220-V500; CD19-PerCP-Cy5.5; CD23-PE, and CD21-APC. Dendritic cell and macrophage panel included: CD11c-APC; CD11b- V450; MHCII- Alexa700. Each mix also included 1% rat serum and 10 μ g Fc γ R blocker (*Fc γ*).

For intracellular cytokine (IC) staining, cells were seeded (2×10^6 cells per well) in 96 well U-bottom plates and stimulated with 50 ng/ml phorbol myristate acetate (PMA) and 250 ng/ml ionomycin in the presence of 200 μ M monensin for 6 h. Following surface staining in 50 μ l antibody mix (CD3-Alexa700, CD4-V500, CD19-PerCP-Cy5.5) for 30 min at 4°C, cells were washed and fixed with 2% paraformaldehyde (PFA) for 30 min in the dark, and permeabilized with 0.1% (m/v) saponin buffer for 30 min. Intracellular cytokines were added for 60 min at 4°C, followed by a wash and final re-suspension in FACS buffer, ready for acquisition. The IC panel contained the following antibodies: IFN- γ -APC; IL-4-PE; IL-10-FITC and IL-13-PeCy7. Acquisition of cells was performed on the Fortessa machine (BD, San Jose, CA, USA). Flow data was analyzed using Flowjo software (Treestar, Ashland, OR, USA).

3.3.6 Enzyme Linked Immunosorbent Assays (ELISAs)

Cytokine secretion in the collected supernatants (Section 3.3.4) was measured by sandwich ELISA, as previously described [233]. For antibody titres, blood was collected in serum

separator tubes (BD Bioscience, San Diego, CA) and serum was separated by centrifugation at 8 000 rpm for 10 min at 4°C. Titres of SEA-specific IgG1, IgG2a, IgG2b, as well as total IgE were determined as previously described [233].

3.3.7 Hydroxyproline assay

Hydroxyproline (OH) content was quantified using a modified protocol [350]. Briefly, weighed liver samples were hydrolysed in 6N HCl and 40mg Dowex/Norit mixture was added. The samples were filtered using Whatmann no. 1 filter paper, neutralised with 1% phenolphthalein and titrated with 10M NaOH and 3N HCl. An aliquot was mixed with isopropanol and chloramine-T/citrate buffer solution (pH 6.5) was added. Erlich's reagent was added and the absorbance was read at 570 nm. OH levels were determined using 4-hydroxy-L-proline as a standard, and the data was expressed as $\mu\text{moles } (\mu\text{M})$ OH per weight of tissue that contained 10^4 eggs.

3.3.8 Histology

Liver and gut samples were fixed in 4% (v/v) formaldehyde in phosphate buffered saline (PBS), embedded in wax, and then processed. Sections (5-7 μm) were cut and stained with hematoxylin and eosin (H&E), periodic acid-Schiff reagent (PAS), and chromotrop aniline blue solution (CAB) counterstained with Wegert's hematoxylin for collagen staining [136]. All the digital images were captured on the Nikon Eclipse 90i DS-Ri2. Liver granuloma diameters were calculated with NIS elements Basic Research software. An average of 20 granulomas per mouse (6 mice per group) was included in the analysis. The number of mucus-producing goblet cells were quantified with Image J software (<https://imagej.nih.gov/ij/>).

3.3.9 Statistics

Statistical analysis was performed using GraphPad Prism 5 software (<http://www.prism-software.com>). The data was calculated as mean \pm SEM. Statistical significance was determined using the unpaired Student's *t* test or 2-way ANOVA with Bonferroni's post-test (unless otherwise stated), defining differences to IL-4R $\alpha^{-/lox}$ as significant (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$).

3.4 Results

3.4.1 KRT14^{cre}IL-4R $\alpha^{-/lox}$ mice infected with *S. mansoni* show survival kinetics and weight loss similar to littermate control

IL-4 and IL-13 signalling is essential for host protection against *S. mansoni* [130, 265, 266, 270, 351]. Given the ability of keratinocytes to signal IL-4 [53, 239], and the ability of cercariae to induce cytokine secretion by keratinocytes [278, 279], we sought to investigate if IL-4/IL-

13 signalling through IL-4R α on keratinocytes would play a role in immunity during schistosomiasis. To determine whether IL-4R α -responsive keratinocytes are required for host survival during acute schistosomiasis, IL-4R $\alpha^{-/lox}$ (littermate control), KRT14^{cre}IL-4R $\alpha^{-/lox}$ and IL-4R $\alpha^{-/-}$ mice were percutaneously infected with 80 live *S. mansoni* cercariae and disease outcome was monitored for 12 weeks. As expected, IL-4R $\alpha^{-/-}$ mice quickly succumbed to infection and all the mice were dead by 7 weeks post-infection (Figure 3.1 A). Conversely, only 20% of KRT14^{cre}IL-4R $\alpha^{-/lox}$ mice had died at 7 week post-infection, similarly to IL-4R $\alpha^{-/lox}$ BALB/c littermate control mice (Figure 3.1 A). However, both KRT14^{cre}IL-4R $\alpha^{-/lox}$ mice and littermate control mice progressively succumbed to infection until 12 weeks post-infection, at which point all mice were dead (Figure 3.1A). We observed significant wasting in IL-4R $\alpha^{-/-}$ mice as indicated by severe body weight loss beginning at 5 weeks post-infection (Figure 3.1 B). No appreciable weight loss was observed in KRT14^{cre}IL-4R $\alpha^{-/lox}$ and littermate control mice (Figure 3.1 B). Therefore, these data indicated that IL-4R α -responsive keratinocytes do not play a role in host protection during acute schistosomiasis in mice.

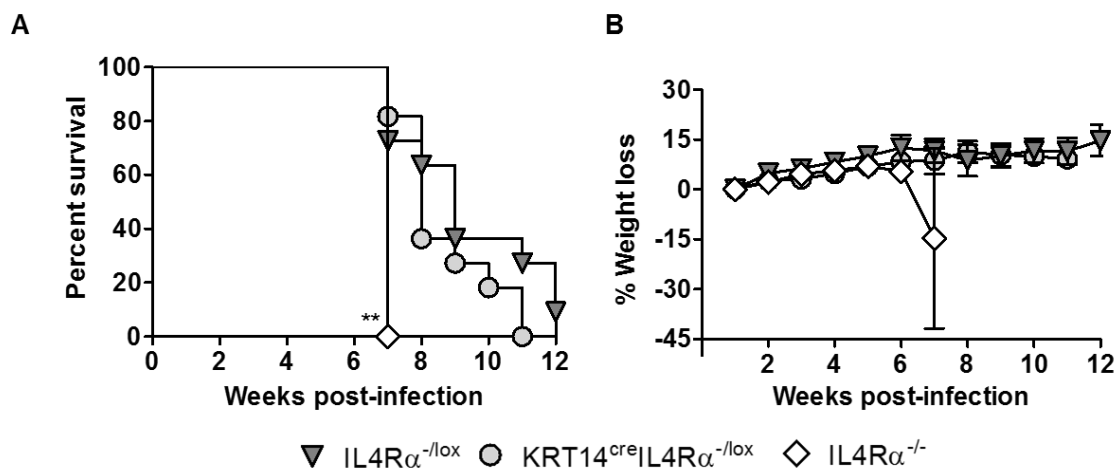


Figure 3.1: IL-4R α responsive keratinocytes are not required for protection against acute schistosomiasis in mice. KRT14^{cre}IL-4R $\alpha^{-/lox}$, IL-4R $\alpha^{-/lox}$ and IL-4R $\alpha^{-/-}$ mice were percutaneously infected with 80 live *S. mansoni* cercariae. (A) Survival kinetics of infected mice monitored over a 12 weeks period. (B) Percent weight loss monitored on a weekly basis. Data are representative of two independent experiments (n = 4-6). Survival curves were compared using Log rank test. **p< 0.01 versus IL-4R $\alpha^{-/lox}$ littermate control mice. IL-4R $\alpha^{-/-}$ mice served as a positive control.

3.4.2 Abrogation of IL-4R α signalling on keratinocytes alters pathology in the liver but not in the gut during acute *S. mansoni* disease

A strong Th2 immune response and granuloma formation occurs in response to *S. mansoni* eggs [134, 265, 266]. In order to investigate the contribution of IL-4/IL-13 responsive keratinocytes on the control of liver and gut pathology, we infected mice with 100 *S. mansoni* cercariae, killed mice at 7 week post-infection (experimental endpoint) and harvested liver and

small intestines for histological examination. We found reduced granuloma sizes in KRT14^{cre}IL-4R α ^{-/lox} mice compared to IL-4R α ^{-/lox} littermate control mice (Figure 3.2 A & D). As expected, IL-4R α ^{-/-} mice had significantly smaller granulomas around the eggs compared to the other strains of mice (Figure 3.2 A). Hydroxyproline content provides a measure of collagen production and is indicative of fibrosis [350]. KRT14^{cre}IL-4R α ^{-/lox} mice and IL-4R α ^{-/lox} mice had comparable hydroxyproline content while IL-4R α ^{-/-} mice had reduced hydroxyproline concentration in the liver (Figure 3.2 B, D). All mutant strains had comparable egg numbers in the liver (Figure 3.2 C & D), indicating that the fitness of the adult worms to lay eggs was not compromised. Mice that quickly succumb to acute schistosomiasis have been shown to suffer from severe gut pathology accompanied by endotoxemia [134]. We investigated whether abrogation of IL-4R α signalling on keratinocytes would impact gut pathology in mice infected with *S. mansoni*. Analysis of H&E stained gut sections showed unaltered gut pathology in all mutant mice (Figure 3.3). However, we observed an increased cellular infiltration in the gut of IL-4R α ^{-/-} compared to the other strains (Figure 3.3). Lastly, we found comparable numbers of mucus producing goblet cells in both KRT14^{cre}IL-4R α ^{-/lox} mice and IL-4R α ^{-/lox} mice, while IL-4R α ^{-/-} had reduced number of goblet cells as depicted by quantification of PAS stained cells in the gut (Figure 3.3 A & B). Together, this data shows that in KRT14^{cre}IL-4R α ^{-/lox} mice, liver granulomas are smaller in comparison to littermate control, while gut inflammation is similar.

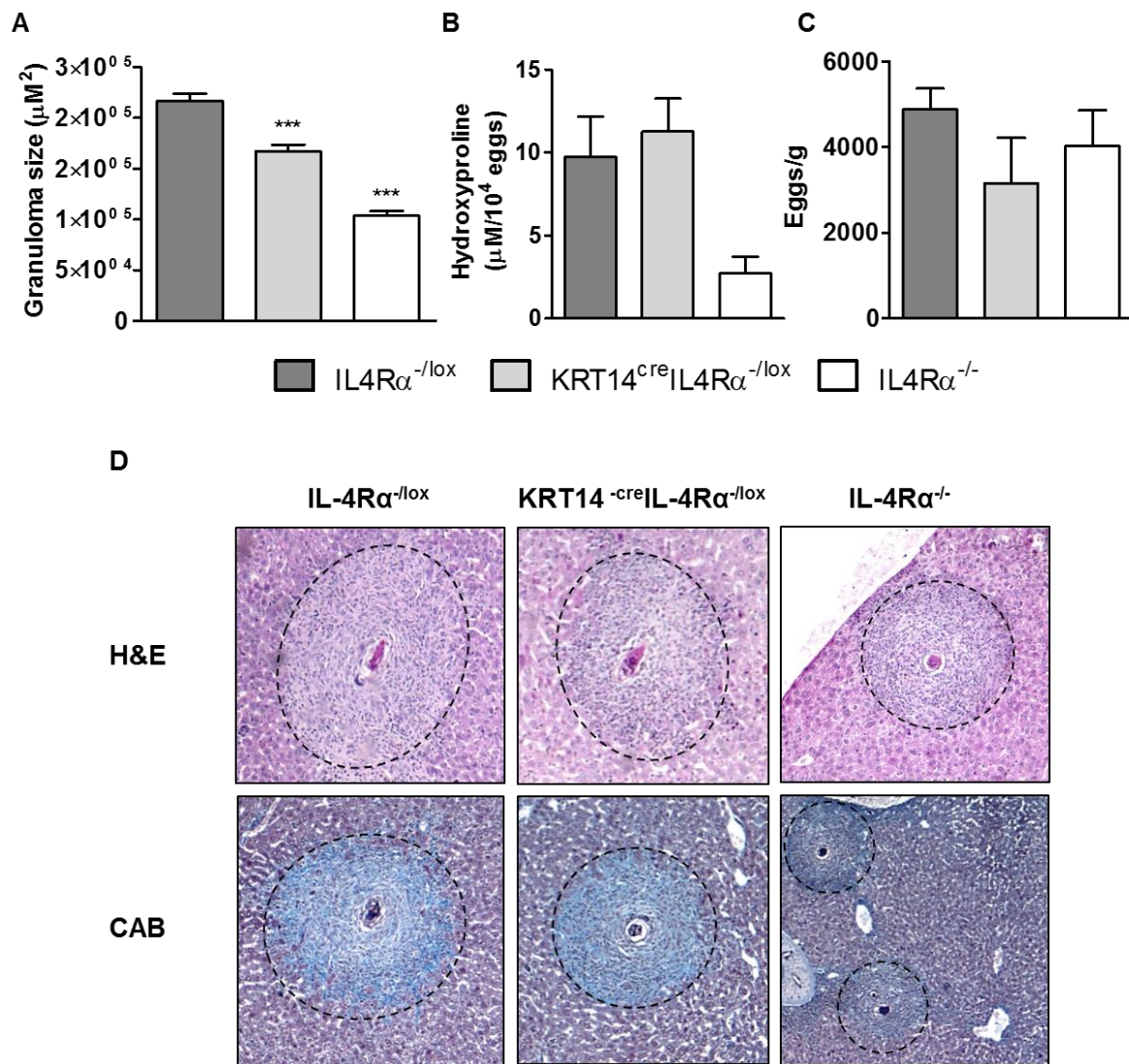


Figure 3.2 Reduced granuloma sizes in KRT14^{cre}IL-4Rα^{-lox} mice during acute schistosomiasis. IL-4Rα^{-lox}, KRT14^{cre}IL-4Rα^{-lox} and IL-4Rα^{-/-} mice were percutaneously infected with 100 live *S. mansoni* cercariae and analysed at 7 weeks post-infection. (A) Granuloma area surrounding eggs quantified by microscopic analysis on H&E stained sections. 20 granulomas per mouse were included in the analysis. (B) Liver fibrosis measured as hydroxyproline normalised to egg numbers. (C) Egg numbers normalised to tissue weight (D) Formalin-fixed liver sections (100x) stained with H&E for morphological analysis, and CAB for collagen content. Data is representative of 2 independent experiments. n= 4-6 mice. ***p< 0.001 vs IL-4Rα^{-lox}.

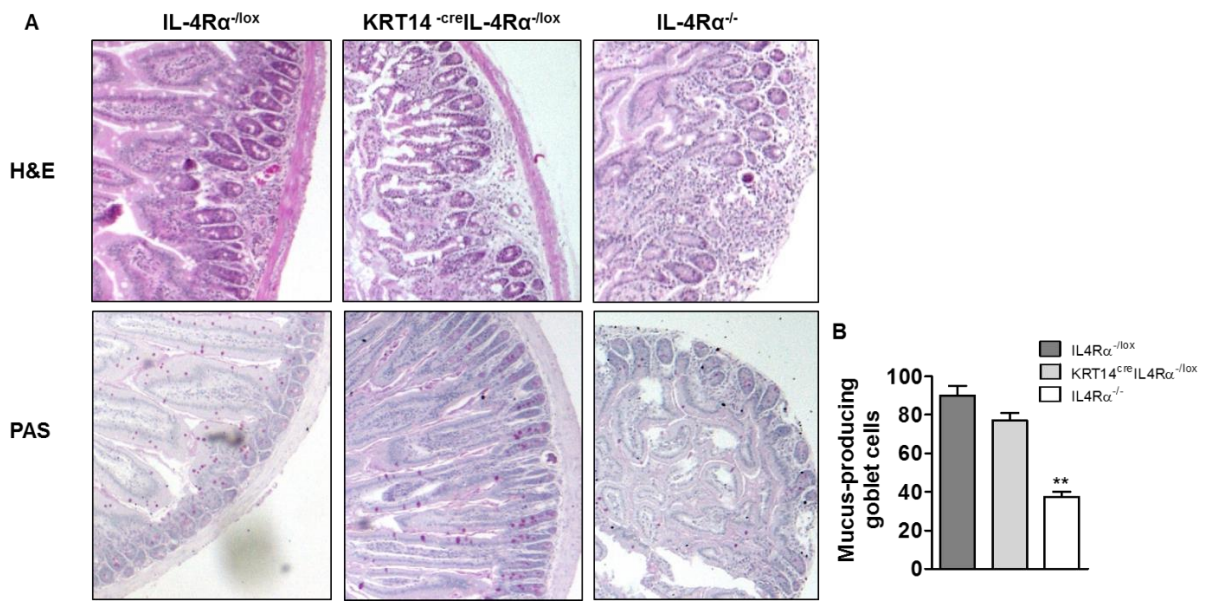


Figure 3.3 Gut pathology of KRT14^{cre}IL-4Rα^{-lox} is unaffected during acute schistosomiasis in mice. KRT14^{cre}IL-4Rα^{-lox}, IL-4Rα^{-lox} and IL-4Rα^{-/-} mice were percutaneously infected with 100 live *S. mansoni* cercariae and analysed 7 weeks post infection. (A) Formalin-fixed liver sections (100x) stained with H&E for morphological analysis and PAS to visualize mucus producing goblet cells. (B) Number of mucus-producing goblet cells present in the small intestine as depicted in the representative image. Data is representative of 2 independent experiments. n= 4-6 mice.

3.4.3 Spleen and liver weights of KRT14^{cre}IL-4Rα^{-lox} mice are characteristic of schistosomiasis

To analyse gross liver and spleen pathology, we determined tissue weight after the organs were harvested from infected mice. We found no significant differences in both the liver and spleen weights of KRT14^{cre}IL-4Rα^{-lox} mice and IL-4Rα^{-lox} littermate control mice (Figure 3.4 A & B). Conversely, infected IL-4Rα^{-/-} mice showed a significant reduction in spleen and liver weights (Figure 3.4A & B), indicating severe multiple organ defects [134]. Taken together, these data suggests that in KRT14^{cre}IL-4Rα^{-lox} mice, spleen and liver organs become enlarged similar to littermate control mice, and as expected during acute schistosomiasis.

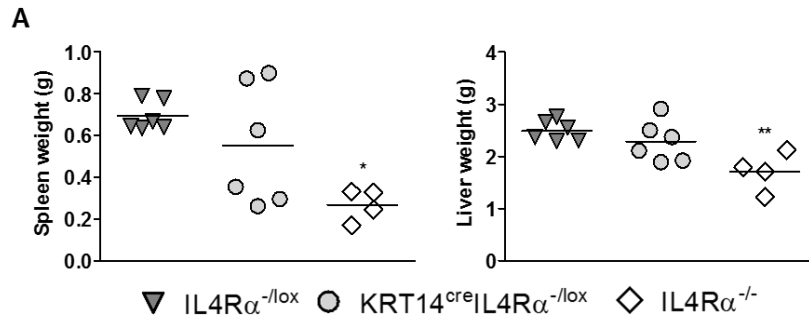


Figure 3.4 Organ weights of KRT14^{cre}IL-4Rα^{-/lox} are unchanged during acute schistosomiasis. IL-4Rα^{-/lox}, KRT14^{cre}IL-4Rα^{-/lox} and IL-4Rα^{-/-} mice were percutaneously infected with 100 live *S. mansoni* cercariae and analysed at 7 weeks post-infection. (A) Spleen weight measured during autopsy. (B) Liver weight measured during autopsy. Data is representative of 2 independent experiments. n= 4-6 mice. *p< 0.05, **p< 0.01 vs IL-4Rα^{-/lox}.

3.4.4 Unaltered *schistosoma*-specific cellular response in infected KRT14^{cre}IL-4Rα^{-/lox} mice

Th2 immune responses is essential for host protective responses during acute schistosomiasis [134, 264-266]. To determine whether *Schistosoma*-specific cellular responses were altered in KRT14^{cre}IL-4Rα^{-/lox} mice, we analysed antigen-specific cytokine responses from mesenteric lymph node (MLN) cells during acute schistosomiasis at 7 weeks post infection (Figure 3.5 A). The abrogation of IL-4Rα expression on keratinocytes did not impair the production of Th2 cytokines (IL-4, IL-5, IL-10 and IL-13) by total MLN cells after stimulation with SEA, similarly to littermate control mice (Figure 3.5 A). The quantity of Th2 cytokines were significantly reduced in IL-4Rα^{-/-} mice compared to littermate control mice (Figure 3.5 A). In contrast, stimulation of total MLN cells from IL-4Rα^{-/-} mice with SEA resulted in increased IFN-γ production, indicating a shift towards a Th1 immune response (Figure 3.5 A). Mitogenic α-CD3 restimulation of MLN cells from KRT14^{cre}IL-4Rα^{-/lox} mice revealed significantly reduced IL-4 production when compared to littermate control mice (Figure 3.5 B). Quantities of IL-5; IL-6; IL-10; IL-13; IFN-γ and IL-17 in KRT14^{cre}IL-4Rα^{-/lox} mice were similar to littermate control (Figure 3.5 B). IL-4Rα^{-/-} mice had significantly less IL-4, IL-5 and IL-10. In addition, IL-4Rα^{-/-} mice seemed to have a higher Th1 to Th2 cytokine ratio in comparison to littermate controls (Figure 3.5 B).

To analyse the impact of IL-4Rα deficiency on keratinocyte on the differentiation and cytokine production by CD4⁺ T cells during *S. mansoni* infection, single cell suspensions were prepared from draining MLN and stained for flow cytometry analysis (Figure 3.5 C & D). There was no significant difference in the percentages of CD3⁺CD4⁺ T helper cells, effector, and central

memory T cells present in the MLN of KRT14^{cre}IL-4R α ^{-/lox} mice compared to littermate control (Figure 3.5 C). In contrast, IL-4R α ^{-/-} mice displayed significantly increased proportions of CD3⁺CD4⁺ T helper, and central memory T cells while the proportion of effector memory was reduced compared to littermate control mice (Figure 3.5 C). Analysis of intracellular cytokine production by CD4⁺ T cells restimulated with PMA/Ionomycin *ex vivo* revealed that MLN cells from KRT14^{cre}IL-4R α ^{-/lox} mice were competent at secreting Th2 cytokines similarly to littermate control mice (Figure 3.5 D). In contrast, we found significantly reduced secretion of both Th1 and Th2 cytokines by cells from IL-4R α ^{-/-} mice as indicated by reduced concentration of IFN- γ , IL-4, IL-13 and IL-10 (Figure 3.5 D). Together these data demonstrate that IL-4R α -responsive keratinocytes are not required for the development of Th2 cellular responses during *S. mansoni* infection in mice.

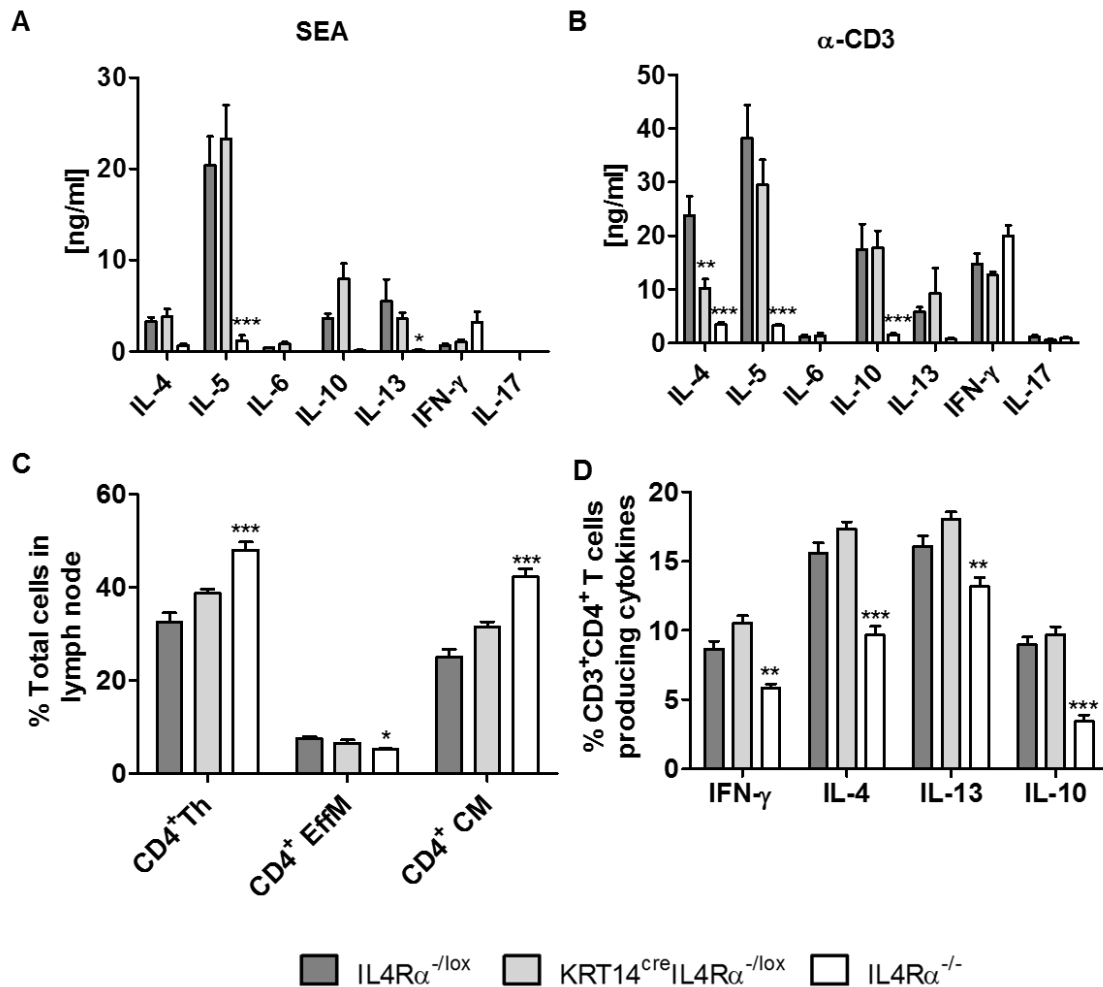


Figure 3.5 Intact cellular response of KRT14^{cre}IL-4Rα^{-/-} during acute schistosomiasis. IL-4Rα^{-/-}, KRT14^{cre}IL-4Rα^{-/-} and IL-4Rα^{-/-} mice were percutaneously infected with 100 live *S. mansoni* cercariae and analysed 7 weeks post-infection. (A-B) Cytokine production by mesenteric lymph node cells restimulated with either SEA or α-CD3. Single cell suspension was prepared from draining mesenteric lymph node (MLN) and cells were stained for flow cytometric analysis. (C) Recruitment of T helper cells (CD3⁺CD4⁺), effector T cells (CD3⁺CD4⁺CD44^{hi}CD62L^{hi}), and central memory T cells (CD3⁺CD4⁺CD44^{hi}CD62L^{hi}) into the secondary lymphoid tissue. (D) Intracellular cytokine detection after restimulation of MLN cells with 25 ng/ml PMA and 1 μg/ml ionomycin *in vitro*. Data are representative of two independent experiments (n = 4-6). Statistical analysis was performed with comparisons to the control IL-4Rα^{-/-} littermate mice group, as significant (*, p<0.05, **, p<0.01; ***, p<0.001). IL-4Rα^{-/-} mice served as a positive control.

3.4.5 Schistosoma-specific humoral responses in infected KRT14^{cre}IL-4Rα^{-/-} mice remained unchanged

B cell responses contribute to the development of a Th2 response during to *S. mansoni* infection [352, 353]. We assessed the impact of IL-4Rα deficiency on keratinocytes on antibody production, by measuring type 1 and 2 antibodies in the serum of infected mice at 7 weeks post infection (Figure 3.6 A-D). KRT14^{cre}IL-4Rα^{-/-} mice had similar titers of both type 1 and 2

antibodies similar to littermate controls (Figure 3.6 A-D). Conversely, global IL-4R α ^{-/-} mice had significantly higher titres of type 1 isotypes IgG2a (Figure 3.6 A & B) and significantly lower titres of type 2 isotypes compared to littermate control mice (Figure 3.6 C & D). The frequencies of CD19⁺B220⁺ B cells, B220⁺CD21⁺CD23⁺ follicular B cells and B220⁺CD21⁺CD23⁻ marginal zone B cells infiltrating the MLN in infected KRT14^{cre}IL-4R α ^{-/lox} mice was similar to littermate controls (Figure 3.6 E). Conversely, infected IL-4R α ^{-/-} mice demonstrated reduced proportions B cells and follicular B cells, and increased marginal zone B cells compared to control mice (Figure 3.6 E). The production of cytokines by B cells were not altered in KRT14^{cre}IL-4R α ^{-/lox} mice similar to littermate control. This was seen in the frequency of IFN- γ and IL-10, as well as similarly higher frequencies of IL-4 and IL-13 (Figure 3.6 F). In contrast, global IL-4R α ^{-/-} mice had significant reduced Th1 and Th2 cytokines secreted by B cells (Figure 3.6 F).

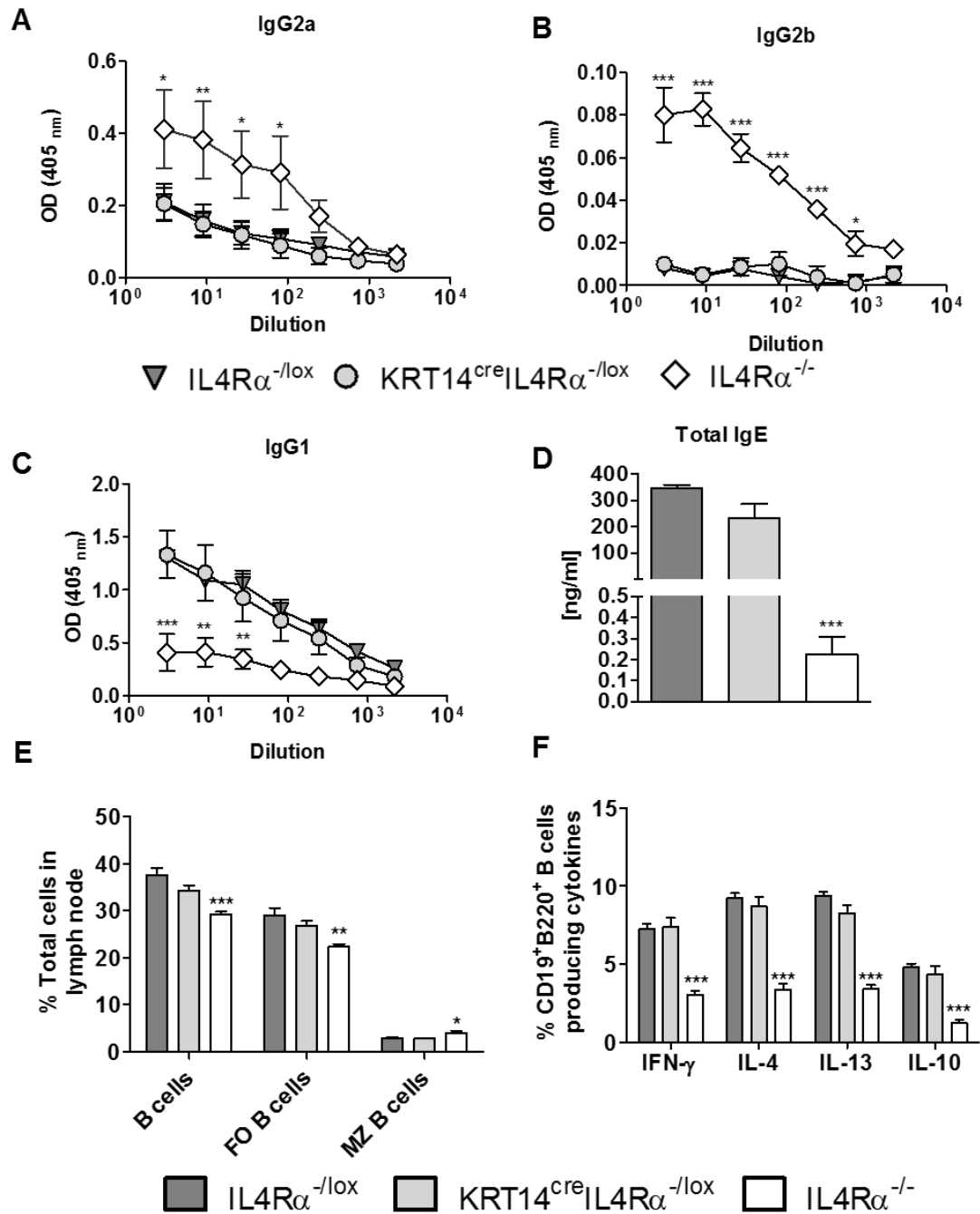


Figure 3.6 Antibody production and lymph node cell B cell-cytokine production in keratinocyte-specific IL-4Rα deficient mice. IL-4Rα^{-/lox}, KRT14^{cre}IL-4Rα^{-/lox} and IL-4Rα^{-/-} mice were percutaneously infected with 100 live *S. mansoni* cercariae and analysed at 7 weeks post-infection. (A-D) SEA specific IgG2a, IgG2b, IgG and total IgE titers were detected in serum by ELISA. Single cell suspensions were prepared from the mesenteric lymph nodes and stained for flow cytometry: (E) recruitment of B cells (CD19⁺B220⁺); (F) follicular B cells (FO, B220⁺CD21⁺CD23⁺), and (G) marginal zone B cells (MZ, B220⁺CD21⁺CD23⁻) into the secondary lymphoid tissue. (H) Histogram showing the expression of activation marker MHCII by B cells. (I) Analysis of intracellular cytokine production by CD19⁺ B cells after restimulation of total MLN cells with 25 ng/ml PMA and 1 μg/ml ionomycin *in vitro*. Data is representative of two experiments (n=4-6). Statistical analysis was performed with comparisons to the control IL-4Rα^{-/lox} littermate mice group, as significant (*, p<0.05, **, p<0.01; ***, p<0.001).

3.5 Discussion

Keratinocytes are the major contributors to the cell population of the skin epidermis [39]. While their involvement in wound healing and dermatitis has been extensively investigated [47, 51, 54, 61, 349, 354, 355], their involvement in immunity in response to infectious disease is still poorly understood [356]. Effective penetration of *S. mansoni* larval stage cercariae through the skin is important to establish infection. Early studies focusing on IL-7 production after *S. mansoni* infection found that keratinocytes were a major source of this cytokine, which contributed to the immune response [278]. Keratinocytes are capable of secreting IL-4 and IL-13, and express the IL-4 receptor mRNA and protein [62, 63, 356]. Furthermore, studies from our laboratory have illustrated the importance of IL-4 and IL-4R α in the development of host protective immune responses during acute schistosomiasis in mice [130, 134, 136, 357]. However, no studies have been carried out to determine the contribution of IL-4/IL-13 signalling through IL-4R α on keratinocytes in the immune response to acute schistosomiasis. We questioned whether abrogation of IL-4R α expression on keratinocytes would affect the development of an immune response and alter the outcome of *S. mansoni* infection. To address this question, our laboratory generated a keratinocyte-specific IL-4R α knock-out mouse model (KRT14^{cre}IL-4R α ^{-/lox}) on a BALB/c genetic background using the Cre/*loxP* system under control of the *KRT14* locus. We found that IL-4R α -responsive keratinocytes are necessary for efficient formation of granuloma structures but are not required for protection during acute schistosomiasis in mice.

A depletion of Th2 responses aggravates tissue damage and increases mortality of the host due to increased pro-inflammatory Th1-type responses [268, 269], as expected in global IL-4R α ^{-/-} mice, that succumbed to *S. mansoni* at 7 weeks post-infection [136]. KRT14^{cre}IL-4R α ^{-/lox} mice developed sufficient Th2 responses to survive up 7 weeks post-infection, and similar to littermate controls, slowly succumbed to infection. This indicated that IL-4/IL-13 signalling through IL-4R α on the keratinocytes were not contributing to host protection during chronic schistosomiasis infection. Wolowczuk *et al.* investigated the cytokine profiles of the skin from mice after *S. mansoni* infection [358]. He detected a maximum quantity of IL-4 at 5 days post infection, but by day 7, this cytokine was undetectable [358]. This early IL-4 could be crucial for educating and priming Th2 immunity that could be required later on for host protection. The presence of the eggs retained within the liver and gut wall causes a Th2-driven response with granuloma formation, which ultimately causes morbidity and mortality in mice and humans [134, 265, 266, 359]. In accordance with this, IL-4R α ^{-/-} mice displayed impaired granuloma formation [134, 275]. KRT14^{cre}IL-4R α ^{-/lox} mice had smaller granulomas in

comparison to the littermate controls. This indicates that while these mice mounted a sufficient Th2 immune response for survival, the response was not optimal for efficient granuloma formation. By blocking the IL-4R α chain, we not only halt signalling of IL-4 through this receptor, but in addition, IL-13 is prevented from signalling through the IL-4R α too. Perhaps this decrease in IL-13, also plays some role in the immune response as IL-13 is involved in the mediation of fibrosis in the liver [276, 360]. Further testing for an indication of hepatocellular damage in these mice by serum measurement of aspartate aminotransferase (AST) could be performed [134].

During *S. mansoni* infection, highly susceptible mice demonstrate increased inflammation of the gut, in addition to endotoxemia and septic shock [134, 265, 275]. In agreement with this, IL-4R $\alpha^{-/-}$ mice demonstrated increased gut inflammation in comparison to littermate control mice. Similar to the littermate control mice, KRT14^{cre}IL-4R $\alpha^{-/lox}$ mice did not show inflammation in the gut, and did not illustrate goblet cell hyperplasia as seen by PAS staining. This suggests that the mice are still able to control gut inflammation and maintain regulatory responses. We did not investigate endotoxemia and septic shock in these mice, but this may be examined by measuring the lipopolysaccharide (LPS) in the serum [130, 134, 136]. Herbert *et al.* demonstrated that IL-4R $\alpha^{-/-}$ mice suffer from defects in their organs, as indicated by severe atrophy of the spleen and liver during acute schistosomiasis [134, 361], supporting our observations in IL-4R $\alpha^{-/-}$ mice in this study. Conversely, KRT14^{cre}IL-4R $\alpha^{-/lox}$ mice demonstrated spleen and liver weights similar to littermate controls, with both mouse groups having exhibited severe splenomegaly, characteristic of *Schistosoma* infection [362, 363].

The development of a polarised Th2 immune response is essential for survival of the host during *S. mansoni* infection [265, 268-270]. Schistosoma-egg antigen (SEA) restimulation of MLN cells from KRT14^{cre}IL-4R $\alpha^{-/lox}$ mice, for cytokine production by total lymph node cell populations, demonstrated a similar response to littermate control mice, both showing a greater Th2 cytokine production. Mitogenic restimulation of MLN cells from KRT14^{cre}IL-4R $\alpha^{-/lox}$ mice demonstrated a similar trend, with the exception of significantly reduced production of IL-4 compared to littermate controls. The production of IL-4 by CD4⁺ T helper cells, however, as determined by intracellular staining, was not downregulated, and illustrated a trend of higher Th2 cytokine production. In agreement with previously published studies, IL-4R $\alpha^{-/-}$ mice demonstrated a shift towards a Th1 immune response, evident in significantly upregulated IFN- γ production after MLN restimulation, and downregulation of Th2 cytokines IL-4, IL-13, IL-5, IL-6 and IL-10 [310]. Differentiation and activation of CD4⁺ T helper cells was unaffected by the absence of IL-4R α -responsive keratinocytes in KRT14^{cre}IL-4R $\alpha^{-/lox}$ mice, similar to

littermate control mice. In contrast, IL-4R α ^{-/-} mice showed significantly reduced effector memory function as previously demonstrated [364], and significantly increased CD4⁺ T helper functions, and memory T cell responses. Contradictory to our findings, Ndlovu *et al.* [364] demonstrated significantly lower CD4⁺ T helper responses in IL-4R α ^{-/-} mice during *S. mansoni* infections. Despite this difference, these mice behaved in the same manner during acute schistosomiasis.

During *S. mansoni* infection, alternatively activated macrophages are recruited to regulate and decrease organ damage that is caused by the trapped eggs [134]. Staining for inducible nitric oxide synthase (for classically activated macrophages) and arginase (for alternatively activated macrophages) could be performed and quantified, to provide an indication of the ratio of classically activated macrophages to alternatively activated macrophages present in KRT14^{cre}IL-4R α ^{-lox} mice.

The B cell and antibody responses contribute to Th2 and immune regulatory responses during *S. mansoni* infection [243, 274]. Antibody responses in KRT14^{cre}IL-4R α ^{-lox} mice demonstrated a predominant type 2 antibody response characterised by the increased production of SEA-specific IgG1 and total IgE, similar to littermate control mice. This indicates that signalling of IL-4 through the IL-4R α on keratinocytes, is not essential in developing sufficient type 2 antibody responses to modulate the immune response. This also indicates that these mice can efficiently undergo antibody isotype switching. Conversely, IL-4R α ^{-/-} mice were seen to have a predominant type 1 antibody response, observed by the increase in production of IgG2a and IgG2b, as expected, and previously described [130, 134, 364]. This finding relates to the role IL-4 plays in isotype switching for the production of type 2 antibodies by B cells [292, 322, 365]. B cell differentiation in KRT14^{cre}IL-4R α ^{-lox} was unchanged, and similar to littermate control mice, confirming these mice can carry out B cell differentiation as normal. In accordance with previous data, IL-4R α ^{-/-} mice had significantly decreased follicular B cells and significantly increased marginal zone B cells, in comparison to the littermate control mice [364]. B cell activation not altered in KRT14^{cre}IL-4R α ^{-lox} mice, similar to littermate controls, while IL-4R α ^{-/-} mice showed a slight decrease as expected [364]. Helminth studies have indicated that B cells can promote Th2 responses, in addition to regulating Th1 responses [352, 366, 367]. Our data suggests that in KRT14^{cre}IL-4R α ^{-lox} mice, B cells are still capable of contributing to the Th2 response, and still has immunomodulatory function. While KRT14^{cre}IL-4R α ^{-lox} mice showed B cell cytokine production to be a mixed Th1/Th2 response, there did appear to be higher levels of Th2 IL-4, IL-13 in comparison to Th1 IFN- γ . The

cytokines secreted by B cells contribute to Th effector functions and memory Th development [368-371]. Given that in KRT14^{cre}IL-4Rα^{-lox} mice, effector and memory Th responses were unchanged, it suggests that sufficient cytokine production by B cells occurs in these mice, independent of IL-4/IL-13 signalling through IL-4Rα on keratinocytes. As expected, global IL-4Rα^{-/-} mice showed significantly decreased Th1 and Th2 B cell cytokines [364]. Together, these findings indicate that IL-4/IL-13 signalling through the IL-4Rα on keratinocytes is not important for the development or differentiation of B cells, or the production of cytokines by these B cells, during *S. mansoni* infection.

3.6 Conclusion

The findings in this study indicate that KRT14^{cre}IL-4Rα^{-lox} mice have a similar mortality rate compared to littermate controls during acute *S. mansoni* infection. In KRT14^{cre}IL-4Rα^{-lox} mice, liver granulomas were smaller than those in littermate controls, however, gut pathology appeared to be similar. Normal hepatosplenomegaly occurred in KRT14^{cre}IL-4Rα^{-lox} mice, similar to littermate control mice. Finally, the Th2 cellular responses, and type 2 antibody responses in KRT14^{cre}IL-4Rα^{-lox} mice were similar to littermate control mice. In the absence of IL-4Rα-responsive keratinocytes, KRT14^{cre}IL-4Rα^{-lox} mice can still elicit a sufficient Th2 response for protection during *S. mansoni* infection. Collectively, our findings indicate that IL-4/IL-13 signalling through the IL-4Rα signalling on keratinocytes is not required for protection during acute *S. mansoni* infection in mice, but does contribute to the optimal formation of Th2 granulomas.

Chapter 4: General Discussion and conclusions

4.1 Aim

The aim of this thesis was to investigate the role of IL-4/IL-13 signalling through IL-4R α on keratinocytes during cutaneous leishmaniasis and acute schistosomiasis. To perform this investigation, our laboratory generated a keratinocyte cell specific-IL-4R α knock-out mouse (KRT14^{cre}IL-4R α ^{-lox}) on both BALB/c and C57BL/6 genetic backgrounds, using gene targeting and site-specific recombination (cre/loxP) under control of the KRT14 promoter.

4.2 Summary of results from Chapter 2

Our work on cutaneous leishmaniasis was presented in Chapter 2. Following footpad infection of KRT14^{cre}IL-4R α ^{-lox} BALB/c mice with *L. major* IL-81 and LV39, we found these mice to remain susceptible to infection, similar to littermate controls. They developed swelling and disease progression, had high parasite burdens, and developed cellular and humoral immunity similar to littermate controls. KRT14^{cre}IL-4R α ^{-lox} C57BL/6 mice infected with *L. major* in the footpad remained resistant to *L. major* infection similar to littermate controls (C57BL/6 genetic background) and C57BL/6 mice. Collectively, our findings indicated that the deletion of the IL-4R α on keratinocytes in BALB/c and C57BL/6 mice did not influence the outcome of disease during *L. major* infection in the footpad model. In the ear infection model, KRT14^{cre}IL-4R α ^{-lox} BALB/c mice infected with *L. major*, developed swelling and disease progression and had high parasite burdens, similar to littermate controls. We did, however, notice that the parasite burden of KRT14^{cre}IL-4R α ^{-lox} BALB/c mice in the ear was significantly lower to littermate control mice, only during infection with *L. major* LV39. The production of Th1 IFN- γ , Th2 IL-4 and IL-13, type 1 IgG2a and IgG2b, and type 2 IgG1 and total IgE were comparable in KRT14^{cre}IL-4R α ^{-lox} BALB/c and littermate control mice. KRT14^{cre}IL-4R α ^{-lox} C57BL/6 mice infected in the ear with *L. major* had decreased swelling and parasite burdens, and developed cellular and humoral similar to littermate control (on C57BL/6 genetic background) and C57BL/6 mice. Together, these results indicated once more, that IL-4R α -responsive keratinocytes do not contribute to the outcome of disease during *L. major* infection in the ear model. From this, we can infer that the IL-4/IL-13, which both signal through the IL-4R α on keratinocytes, are not required for control of infection during cutaneous leishmaniasis. Additionally, on a BALB/c background, the lack of IL-4R α -responsive keratinocytes allows these mice to better control the replication of parasites at the site of infection compared to littermate control, indicating that the IL-4/IL-13 signalling through the IL-4R α on keratinocytes appeared to contributed to susceptibility in these mice.

4.3 Studies contributing to the generation of the hypothesis in Chapter 2

Our work followed on from two pivotal studies on *L. major* by Biedermann *et al.* [235] and Ehrchen *et al.* [239] that are extensively described in Chapter 2 of this thesis. Briefly, Biedermann *et al.* illustrated the “IL-4 instruction theory” showing that recombinant IL-4 administered exogenously can instruct dendritic cells to secrete IL-12 to drive a T helper 1 (Th1) polarised immune response [235]. This was demonstrated with *in vitro* and *in vivo* mouse experiments. They also found that a push towards a Th1 immune response required IL-4 that was present at the early stages of infection. The findings presented by Biedermann *et al.* [235] were also recently supported by work carried out in our laboratory by Hurdayal *et al.* [135] who provided a better indication of the contribution of biological quantities of endogenous IL-4 to drive dendritic cells to elicit a polarised Th1 immune response. Here, the authors carried out *L. major* infections on genetically modified BALB/c mice, having abrogated IL-4 receptor alpha (IL-4R α) on dendritic cells. Their findings indicated that IL-4-mediated instruction of dendritic cells occurs *in vivo* with biological quantities of IL-4 acting on IL-4R α -responsive dendritic cells to promote survival of the host [135], and further supported the findings reported by Biedermann *et al.* [235]. Working on the theory that early IL-4 is vital in driving dendritic cells to induce a Th1 immune response, Ehrchen *et al.* directed the focus to the early stages of infection, and investigated the initial infected site, the microenvironment of the infected tissue [239]. They found that keratinocytes are rapidly induced to secrete immunomodulatory mediators IL-12, IL-1 β , and IL-4, in the presence of *L. major* promastigotes, and Th2 cytokine IL-4 from keratinocytes appeared to be the significantly highly present in genetically resistant C57BL/6 mice. Additionally, early local neutralization of the IL-4 in these resistant mice resulted in a Th2 switch, making these mice susceptible to *L. major* infection [239]. These finding suggested a possible role for keratinocytes in contributing to Th1 immunity during experimental leishmaniasis. Collectively, the data and observations from Biedermann *et al.* [235] and Ehrchen *et al.* [239] support a possible role for keratinocyte-derived-IL-4 immunity against *Leishmania*. From these studies, we constructed a hypothesis: if early IL-4 instructing dendritic cells is derived from keratinocytes, and we deleted the IL-4R α signalling receptor through which IL-4 signals on these cells, then IL-4 will not be able to signal in this manner, and dendritic cells would not be able to promote a Th1 response. While Ehrchen *et al.* did not find IL-13 to be significant during his study [239], we could not exclude IL-13, as this cytokine can signal through the IL-4R α signalling receptor on keratinocytes [67].

4.4 Considerations (*L. major* study)

The footpad infection model for leishmaniasis is well established in our laboratory [132, 135, 294] and in other groups [285-287]. Furthermore, both Biedermann *et al.* [235] and Ehrchen *et al.* [239] used this model for their experiments. For these reasons, we performed experiments using this model. When we found no significant findings using the footpad infection model, we decided to test our hypothesis using the ear infection model. The ear infection model involves an intradermal inoculation of low dose metacyclic promastigote *L. major* parasites in the ear, a site of infection that is becoming more favoured, as it better mimics natural infection by the sandfly, and is better at reproducing the natural lesion formed [281-283, 326-329]. In addition to these reasons, considering we did find a significant reduction of parasite burden in the ear of infected KRT14^{cre}IL-4Rα^{-lox} BALB/c mice, this model may in fact be a better choice for *L. major* infections.

Further experiments could be performed to strengthen our findings. As Ehrchen *et al.* looked at the skin after infection with *L. major* [239], we could perform similar experiments to further investigate the site of infection, in both the footpad and ear model. Here we could observe cellular infiltration by extracellular staining on the skin, and skin-draining lymph nodes to look at other cells that may be contributing to the immune response or control of infection. Furthermore, the question of the source of early IL-4 driving dendritic cells to develop a polarised Th1 response has yet to be determined. To address this, neutrophils and mast cells could be explored further, as described in Chapter 2. These cells can signal IL-4/IL-13 through IL-4Rα, and are two of the first cells to make contact with the *Leishmania* parasites after infection [183-185, 331, 334, 339, 341]. Additionally, eosinophils and basophils could be investigated [372]. During *L. major* and *L. amazonensis* infection, eosinophils are recruited to the site of infection [373, 374]. Furthermore, eosinophils have been suggested to contribute to parasite clearance at the site of infection, during the initial hours of *L. mexicana* infection [375]. In humans, basophils are a key source of IL-4, which contributes to pathogenesis during allergic inflammation [376, 377]. These cells are also an important source of IL-13 [372, 376, 378]. It would be interesting to determine if these cells contribute to *L. major* infection. Histological analysis of the infected footpad or ear could be performed to observe the sites of infection, and changes in epithelial structure. Our intracellular staining panels could also include markers to indicate the presence and proportions of classically and alternatively activated macrophages in our KRT14^{cre}IL-4Rα^{-lox}. Additionally, we need to consider, that if IL-13 is playing a role during *L. major* infection, this cytokine can signal not only through the IL-4Rα on keratinocytes, but also through the IL-13R on these cells.

4.5 Conclusions to *L. major* study

Overall, we showed that IL-4R α signalling on keratinocytes does not drive a Th1 immune response and is not important for control during *L. major* infection. From this, we infer that IL-4/IL-13 signalling through the IL-4R α receptor on keratinocytes also does not influence the immune response, and does not drive Th1 immunity as suggested. We demonstrated this using the footpad and ear models for *L. major*. Our findings also highlight the importance of noting the difference in source and sub strain of *L. major* parasite during experiments, as this difference can alter the immune response differently. Finally, we have provided the first report on the immune responses in global IL-4R $\alpha^{-/-}$ on the C57BL/6 genetic background during *L. major* infection in mice.

4.6 Chapter 3: Hypothesis, summary and conclusions from *S. mansoni* study

Schistosomiasis is a Th2 controlled disease, while leishmaniasis is Th1 controlled, and while IL-4R α is important for host protection during acute schistosomiasis as shown with mouse models [130, 134, 136, 277], IL-4R α plays a role in susceptibility to leishmaniasis as previously explained. The skin is involved in the life cycle of the *Schistosoma* parasite, as explained in Chapter 1, and the epidermal layer is abundant in keratinocytes. Furthermore, it has been shown *S. mansoni* cercariae can influence keratinocytes to secrete IL-7 in response to *S. mansoni* infection [278, 279]. Keeping these facts in mind, we hypothesised that IL-4R α -responsive keratinocytes would play a role in protection during *S. mansoni* infection. We tested this hypothesis using the KRT14^{cre}IL-4R $\alpha^{-/lox}$ BALB/c mouse. After the mice were percutaneously infected with *S. mansoni* cercariae, we monitored disease progression and evaluated their immune response. Mortality studies indicated that IL-4R α -responsive keratinocytes did not appear to increase protection in these mice during chronic *S. mansoni* infection, as they behaved similar to littermate control mice, indicated by survival and weight loss. The development of a polarised Th2 immune response is required for the hosts' survival during *S. mansoni* [265, 268-270]. The production of T helper cytokines in KRT14^{cre}IL-4R $\alpha^{-/lox}$ BALB/c mice appeared to be Th2 polarised and was similar to littermate control mice. During *S. mansoni* infection, immunomodulation is necessary for protection, and mechanisms include B cell and antibody responses [243, 274]. KRT14^{cre}IL-4R $\alpha^{-/lox}$ mice demonstrated a predominant type 2 antibody response as seen by the increased production of IgG1 and total IgE, similar to littermate control mice. We noted that in these KRT14^{cre}IL-4R $\alpha^{-/lox}$ BALB/c mice, the areas of the granulomas were significantly less compared to littermate control mice, suggesting a role for IL-4R α -responsive keratinocytes in granuloma formation. Overall, IL-

4/IL-13 signalling the IL-4R α on keratinocytes does not appear to be important in the control of disease during *S. mansoni* infection.

4.7 Considerations (*S. mansoni* study)

As with the *Leishmania* study, the role of IL-13 should be considered, both IL-4R α -dependent and IL-13R dependent, as IL-13 does contribute to Th2 immunity during schistosomiasis. Additional experiment could be performed to supplement our findings. It would be interesting to also investigate the infection site (the abdominal skin where the cercariae penetrate), and the skin-draining lymph nodes of KRT14^{cre}IL-4R α ^{-/lox} mice shortly after infection, implementing histological staining, extracellular staining for cell population, and cytokine assays. This can illustrate any early effects of the deletion of IL-4R α -responsive keratinocytes during acute schistosomiasis. Liver pathology could be investigated for the presence of classically or alternatively activated macrophages by the presence iNOS and Arginase, by histological staining of sections of the liver. To provide an indication of hepatocellular damage in KRT14^{cre}IL-4R α ^{-/lox} mice, the levels of aspartate transaminase in the serum could be measured. Gut pathology could also be looked at further, by detection of LPS in the serum of KRT14^{cre}IL-4R α ^{-/lox} mice.

4.8 Final conclusions

IL-4/IL-13 signalling through the IL-4R α on keratinocytes in BALB/c and C57BL/6 mice is not essential for control of disease during *L. major* infection in mice. IL-4/IL-13 signalling through the IL-4R α on keratinocytes in BALB/c mice contribute to granuloma formation, but are not required for the control of acute schistosomiasis.

4.9 Relevance of the studies

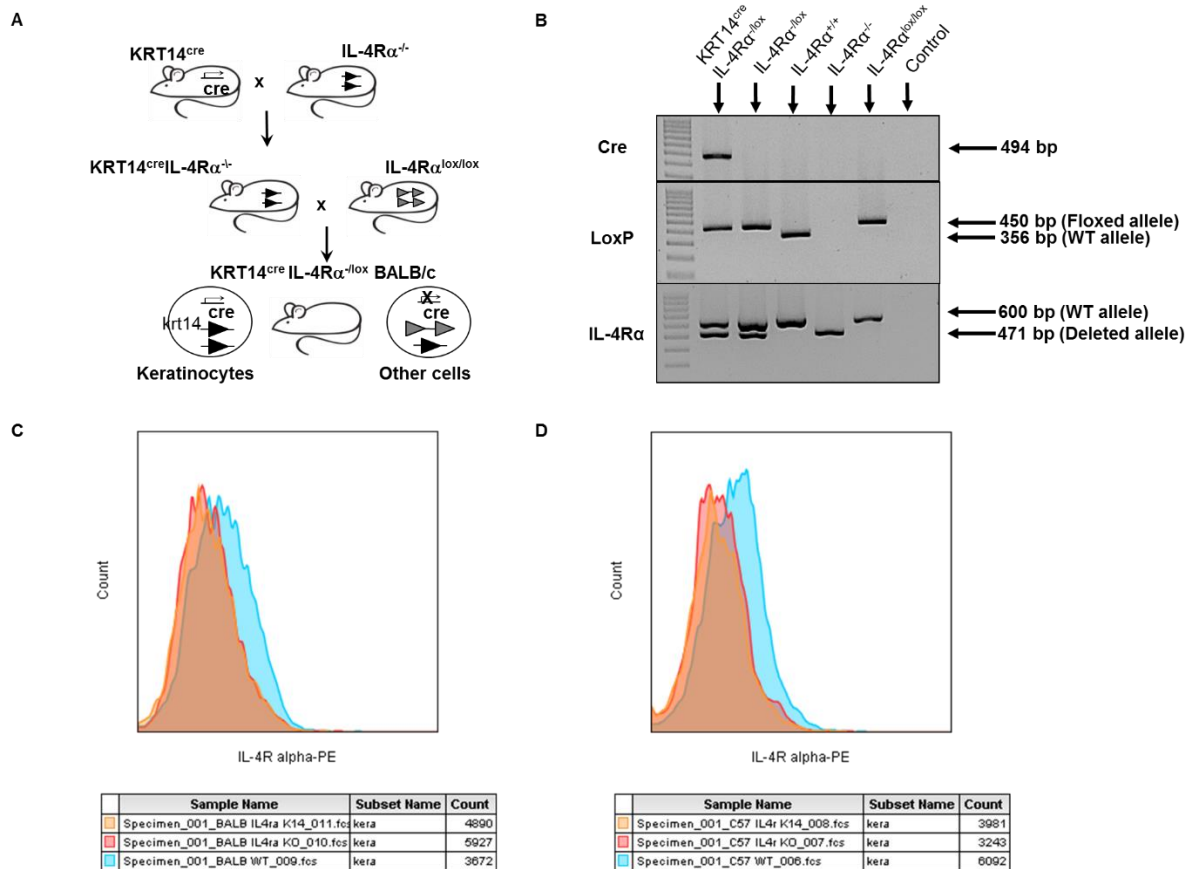
Neglected tropical diseases (NTD) occur in 149 countries worldwide, primarily in the tropical and subtropical regions [379]. There are currently 17 different diseases characterised as an NTD, including Echinococcosis, human African Trypanosomiasis, leishmaniasis, and schistosomiasis [379, 380]. Factors contributing to spread of these diseases include poverty, inadequate sanitation, and close proximity to infectious vectors and domestic livestock [379, 380]. While there are control strategies that have been set in place, for many of these diseases control alone is not sufficient, and treatment with drugs and vaccines is crucial [379-382]. Furthermore, for leishmaniasis and schistosomiasis, despite numerous vaccine targets and trials, there has yet to be a safe and effective vaccine designed. Due to their high morbidity and mortality rates, cases of drug toxicity and increased drug resistance [382, 383], understanding

underlying mechanisms of host-parasite interactions, pathogenesis, and immune responses is extremely important. While some of these studies, like ours, may not provide drug or vaccine targets, they still raise important questions and ideas which could contribute in some way to the fight against these diseases.

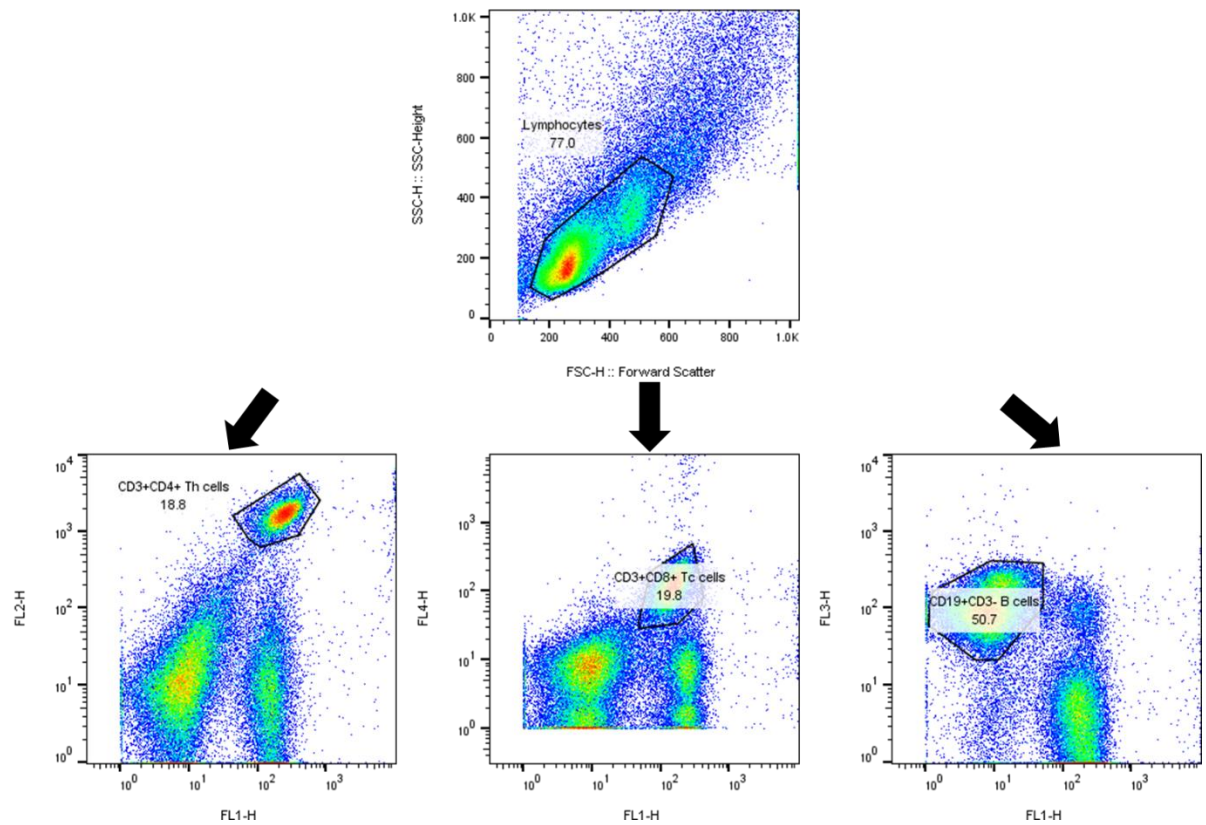
4.10 Future work: wound healing studies

The skin is regarded the first line barrier of protection in humans and animals, and contains cells in the dermis and epidermis. The epidermis is stratified epithelium that contains of ten to twenty layers of keratinocytes. These cells make up majority of the cell population in the epidermis [39]. Apart from their role in barrier maintenance, keratinocytes play an important role in wound healing, and are essential during the epithelialization phase of this process [47]. Furthermore, keratinocytes are capable of producing growth factors, cytokines, and chemoattractants, as described in detail in Chapter 1 [53]. There are many factors that hamper wound healing. These factors may be regarded as either local or systemic. Local factors such as oxygenation, infection and foreign agents, influence the characteristics of the wound directly. Systemic factors such as age, gender, stress, obesity and cancer, represents the general health or diseased state of the individual that alters their ability to heal the wound. The importance of keratinocytes in skin-related diseases including atopic dermatitis and allergy has also been highlighted [54, 384]. IL-4 and IL-4R α have been shown to be involved in wound healing [354, 385-387]. A study of note was performed by Salmon-Ehr *et al.*, who detected IL-4 early at the site of a wound, and not in non-wounded controls [354]. Additionally, he showed accelerated wound closure in mouse wounds treated with IL-4, when compared to those treated with PBS controls, further implying that IL-4 is beneficial for wound-healing [354]. We hypothesize that IL-4/IL-13 signalling through the IL-4R α on keratinocytes may play a role in wound-healing, and aim to further investigate this with a wound healing model based on the KRT14^{cre}IL-4R α ^{-/lox} mouse.

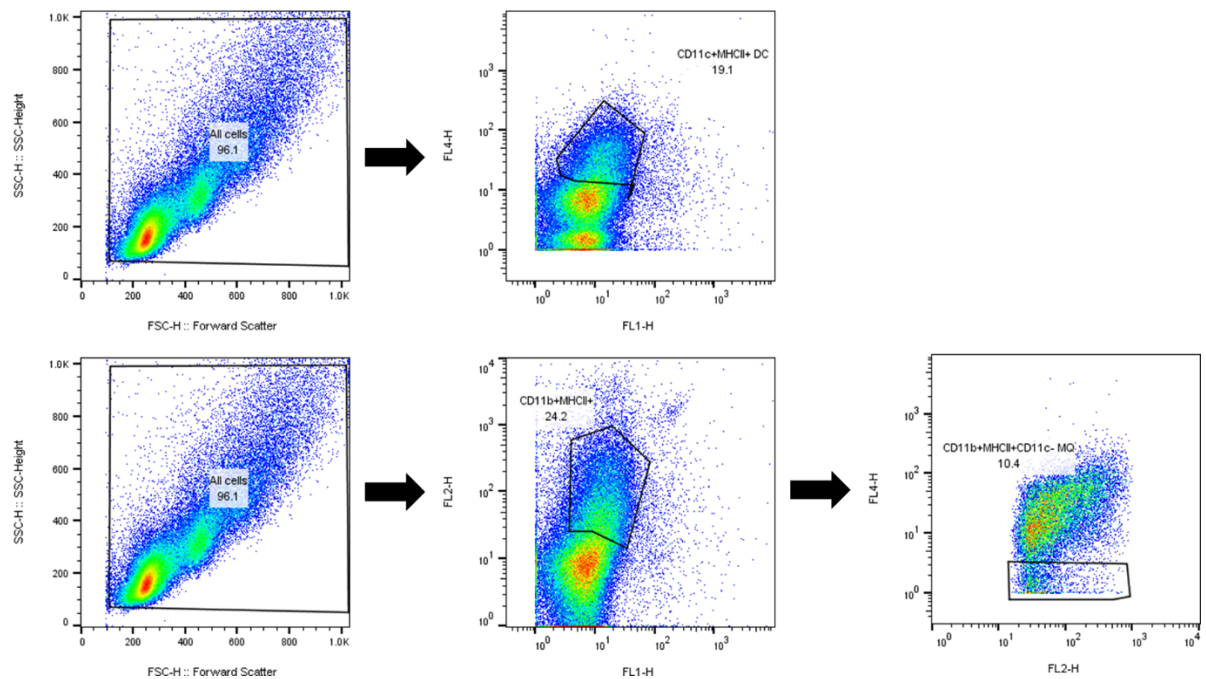
Supplementary figures



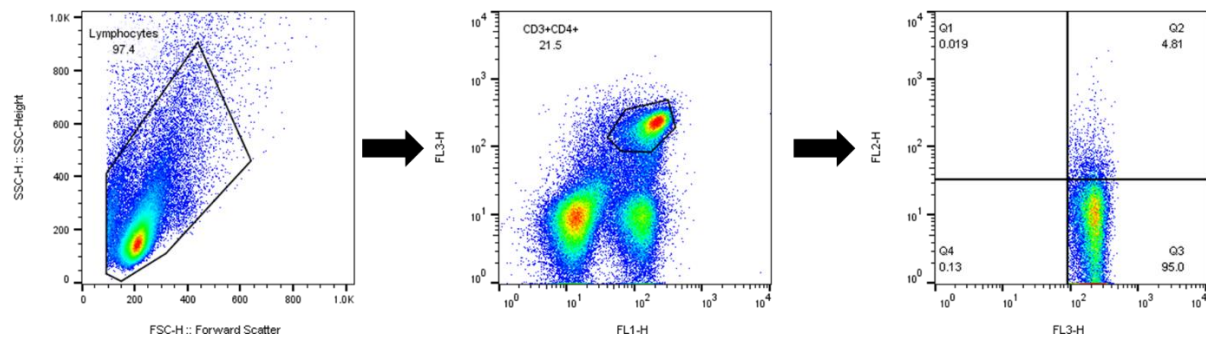
Supplementary Figure 1. Characterization of KRT14^{cre}IL-4R α ^{-lox} mice. (A) Mouse breeding strategy. Transgenic BALB/c mice expressing Cre-recombinase under control of the krt14 promoter was inter-crossed with IL-4R α ^{-/-} BALB/c mice and IL-4R α ^{lox/lox} BALB/c mice to generate Lck^{cre}IL-4R α ^{-lox} mice (B) PCR analysis of KRT14^{cre}IL-4R α ^{-lox} on a BALB/c and C7BL/6 background indicated the following: KRT14^{cre} specific gene is 494 base pairs (bp), loxP is 450 base pairs (floxed) or 356 base pairs (wildtype) and deleted IL-4R α gene is 471 bp, with the wild-type (WT) IL-4R α being 600 bp. (C-D) Phenotypic analysis. Flow cytometry was performed to show IL-4R α expression on isolated ear keratinocytes in KRT14^{cre}IL-4R α ^{-lox} on BALB/c (C) and C57BL/6 genetic backgrounds (D): KRT14^{cre}IL-4R α ^{-lox} (orange), global IL-4R α ^{-/-} (red), and wildtype BALB/c mice (blue). Keratinocytes were gated as CD45⁻CD49⁺K14⁺. Inter-crossing and genotyping of mice was performed by the UCT Genotyping team. PCR analysis was performed by Miss M. Ansarie of the UCT Genotyping team. Isolation of ear keratinocytes and IL-4R α staining was performed at the University Of Lausanne, in the Tacchini-Cottier laboratory by the author, assisted by Dr. M. Descatoire.



Supplementary Figure 2. Flow cytometry plots and gating strategies for surface staining of lymph node cells to illustrate T helper, T cytotoxic and B cell populations.



Supplementary Figure 3. Flow cytometry plots and gating strategies for surface staining of lymph node cells to illustrate dendritic cell and macrophage populations.



Supplementary Figure 4. Flow cytometry plots and gating strategies for surface staining of lymph node cells to illustrate T helper cytokine production. Q2 denotes the CD3⁺CD4⁺ cytokine.

Appendix I: List of media and buffers

Media/Buffer	Composition
10× Phosphate buffered saline (PBS)	Dissolve 80g NaCl, 2g KCl, 14.4g H ₂ PO ₄ , 2.4g KH ₂ PO ₄ in 1 L distilled H ₂ O.
Schneider's medium (SCH+FCS)	Dissolve 1 x bottle Schneider's Drosophila Medium (Sigma-Aldrich) and 0.4 g Sodium Bicarbonate in 800 ml distilled H ₂ O. Adjust pH to 9.2. Stir (10 min), adjust pH to 6.7. Dissolve 0.6 g anhydrous calcium chloride in 50 ml water. Add slowly to medium. Add 20 % heat-inactivated FCS. Adjust pH to 7.0. Add 2.5 ml 100 U/ml penicillin G and 100 µg/ml streptomycin (Pen/Strep). Make volume up to 1 L with distilled H ₂ O, filter-sterilize.
Complete Dulbecco's Modified Eagle's Medium (cDMEM)	Add 50 ml % FCS, 5 ml HEPES, 500 µl β-2-mercaptoethanol, 2.5 ml Pen/Strep to 442 ml DMEM (Gibco)
Compete Iscove's Modified Dulbecco's Medium (IMDM)	Add 2.5 ml Pen/Strep, 50 ml FCS, to 447,5 ml IMDM (Gibco), adjust pH to 7.4
FACS Buffer	Add 1 g bovine serum albumin (BSA) to 1 L 1× PBS, filter-sterilize
Permeabilization Buffer	Add 0.5 g Saponin, 0.055 g CaCl ₂ , 0.0625 g MgSO ₄ , 0.25 g NaN ₃ , 0.5 g BSA, 5 ml 1 M HEPES to 400 ml 1× PBS. Adjust to pH 7.4. Make volume up to 500 ml with distilled H ₂ O, filter-sterilize.
ELISA Blocking Buffer	Dissolve 20g Milk powder (spar instant) in 1 L 1× PBS
Cytokine ELISA Carbonate Coating Buffer	Add 1.6 g Na ₂ CO ₃ , 2.9 g NaHCO ₃ , 4.2 g NaCl to 100 ml 10× PBS. Make volume up to 1 L with distilled H ₂ O, adjust pH to 9.5
Antibody ELISA Coating Buffer	Dissolve 8g BSA (Merck) in 100 ml 10× PBS. Adjust pH to 9.5, make volume up to 1 L in with distilled H ₂ O,
ELISA Dilution Buffer	Dissolve 10g BSA (Roche), 0.2g NaN ₃ (Merck). Make volume up to 1 L with 1× PBS
ELISA Substrate Buffer	Dissolve 0.2g NaN ₃ , 97 ml di-ethanolamine, 0.8g MgCl ₂ .6H ₂ O in 700 ml distilled H ₂ O, adjust pH to 9.8. Make volume up to 1 L with distilled H ₂ O

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